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(54) Title: TNF–RELATED PROTEINS

(57) Abstract

A member of the tumor necrosis factor family and associated antibodies and uses are described. This member is primarily expressed in B cells and its expression correlates to increases in the number of B cells and immunoglobulins produced. The human ortholog contains 285 amino acids; the mouse ortholog, 309 amino acids. The protein is a type II transmembrane protein and has an amino terminal cytoplasmic domain, a transmembrane domain, and a carboxy terminal extracellular domain. TNF–related proteins of the invention may be membrane–associated or in soluble form, recombinantly produced or isolated after natural production. The invention provides for nucleic acids encoding such TNF–related proteins, vectors and host cells expressing the polypeptides, and methods for producting recombinant porteins. Antibodies, fragments, and related fusion proteins and derivatives may be used as agonists or antagonists of AGP–3 related activity.

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TNF-RELATED PROTEINS

Cross-reference to Related Applications

This specification is related to U.S. provisional application nos. 60/119,906, filed February 12, 1999 and 60/166,271, filed November 18, 1999, respectively, both of which are hereby incorporated by reference in their entirety.

Field of the Invention

The present invention relates to proteins that are involved in inflammation and immunomodulation, particularly in B cell growth, survival, or activation. The invention further relates to proteins related to the tumor necrosis factor (TNF)/nerve growth factor (NGF) superfamily and related nucleic acids, expression vectors, host cells, and binding assays. The specification also describes compositions and methods for the treatment of immune-related and inflammatory, autoimmune and other immune-related diseases or disorders, such as rheumatoid arthritis (RA), Crohn's disease (CD), lupus, and graft versus host disease (GvHD).

The invention also relates to methods and compositions for the treatment of inflammatory and immune-related diseases and disorders using the receptors.

Background of the Invention

After years of study in necrosis of tumors, tumor necrosis factors (TNFs) α and β were finally cloned in 1984. The ensuing years witnessed the emergence of a superfamily of TNF cytokines, including fas ligand (FasL), CD27 ligand (CD27L), CD30 ligand (CD30L), CD40 ligand (CD40L), TNF-related apoptosis-inducing ligand (TRAIL, also designated AGP-1), osteoprotegerin binding protein (OPG-BP or OPG ligand), 4-1BB ligand, LIGHT, APRIL, and TALL-1. Smith et al. (1994), Cell 76: 959-962; Lacey et al. (1998), Cell 93: 165-176; Chichepotiche et al. (1997), J. Biol.

Chem. 272: 32401-32410; Mauri et al. (1998), Immunity 8: 21-30; Hahne et al. (1998), J.Exp. Med. 188: 1185-90; Shu et al. (1999), J. Leukocyte Biology 65: 680-3. This family is unified by its structure, particularly at the C-terminus. In addition, most members known to date are expressed in immune compartments, although some members are also expressed in other tissues or organs, as well. Smith et al. (1994), Cell 76: 959-62. All ligand members, with the exception of LT-α, are type II transmembrane proteins, characterized by a conserved 150 amino acid region within the C-terminal extracellular domain. Though restricted to only 20-25% identity, the conserved 150 amino acid domain folds into a characteristic β-pleated sheet sandwich and trimerizes. This conserved region can be proteolyticaly released, thus generating a soluble functional form. Banner et al. (1993), Cell 73: 431-445.

Many members within this ligand family are expressed in lymphoid enriched tissues and play important roles in immune system development and modulation. Smith et al. (1994). For example, TNFα is mainly synthesized by macrophages and is an important mediator for inflammatory responses and immune defenses. Tracey & Cerami (1994), Annu. Rev. Med. 45: 491-503. Fas-L, predominantly expressed in activated T cell, modulates TCR-mediated apoptosis of thymocyts. Nagata, S. & Suda, T. (1995) Immunology Today 16: 39-43; Castrim et al. (1996), Immunity 5: 617-27. CD40L, also expressed by activated T cells, provides an essential signal for B cell survival, proliferation and immunoglobulin isotype switching. Noelle (1996), Immunity 4: 415-9.

The cognate receptors for most of the TNF ligand family members have been identified. These receptors share characteristic multiple cysteine-rich repeats within their extracellular domains, and do not possess catalytic motifs within cytoplasmic regions. Smith et al. (1994). The receptors signal through direct interactions with death domain proteins (e.g. TRADD, FADD, and RIP) or with the TRAF proteins (e.g.

TRAF2, TRAF3, TRAF5, and TRAF6), triggering divergent and overlapping signaling pathways, e.g. apoptosis, NF-κB activation, or JNK activation. Wallach et al. (1999), Annual Review of Immunology 17: 331-67. These signaling events lead to cell death, proliferation, activation or differentiation. The expression profile of each receptor member varies. For example, TNFR1 is expressed on a broad spectrum of tissues and cells; whereas the cell surface receptor of OPGL is mainly restricted to the osteoclasts. Hsu et al. (1999) Proc. Natl. Acad. Sci. USA 96: 3540-5. It is therefore an object of the invention to identify proteins and nucleic acids related to TNFs. Such proteins are believed to play a role in inflammatory and immune processes, suggesting their usefulness in treating autoimmune and inflammatory disorders.

Summary of the Invention

In accordance with the present invention, the inventors describe a novel member of the tumor necrosis factor family. The novel TNF ligand family member is herein called AGP-3. Unlike other members of the family, the receptor for AGP-3 is primarily expressed in B cells, and its expression correlates to increases in the number of B cells and immunoglobulins produced.

The natural, preferred human ortholog is here called hAGP-3 and contains 285 amino acids; the mouse ortholog (mAGP-3), contains 309 amino acids. The AGP-3 protein is a type II transmembrane protein and has an amino terminal cytoplasmic domain, a transmembrane domain, and a carboxy terminal extracellular domain. TNF-related proteins of the invention may be membrane-associated or in soluble form, recombinantly produced or isolated after natural production. The present specification demonstrates that AGP-3 is a potent B cell stimulatory factor. Interestingly, the AGP-3 transgenic mice also developed autoantibodies

and kidney immune complex deposits, a phenotype resembling lupus patients and lupus prone mice.

The invention provides for nucleic acids encoding AGP-3, vectors and host cells expressing the polypeptides, and methods for producing recombinant proteins. Antibodies or fragments thereof that specifically bind AGP-3 are also provided.

The subject proteins may be used in assays to identify cells and tissues that express AGP-3 or proteins related to AGP-3 and to identify new AGP-3-related proteins. Methods of identifying compounds that interact with AGP-3 proteins are also provided. Such compounds include nucleic acids, peptides, proteins, carbohydrates, lipids or small molecular weight organic molecules and may act either as agonists or antagonists of AGP-3 or AGP-3 R-protein activity.

AGP-3-related proteins are involved in B cell growth, survival, and activation, particularly in lymph node, spleen, and Peyer's patches. AGP-3 agonists and antagonists (e.g., antibodies to AGP-3) thus modulate B cell response and may be used to treat diseases characterized by inflammatory processes or deregulated immune response, such as RA, GvHD, CD, lupus, and the like. Pharmaceutical compositions comprising AGP-3-related proteins and AGP-3 agonists and antagonists are also encompassed by the invention.

In addition to therapeutic applications, AGP-3 related proteins may also be useful in production of hybridoma cells, which are derived from B cells. Thus, the present invention also concerns a method to modulate hybridoma cell antibody production, which comprises treating hybridoma cells with the compound of Claim 1.

Description of the Figures

Figure 1 shows the sequence of human AGP-3. Nucleic acid and amino acid sequences of human AGP-3 are indicated (SEQ ID NOS: 1 and

2, respectively). The predicted transmembrane region is underlined. Potential N-linked glycosylation sites are shown in boldface.

Figure 2 shows the sequence of murine AGP-3. Nucleic acid and amino acid sequences of murine AGP-3 are indicated (SEQ ID NOS: 3 and 4, respectively). The predicted transmembrane region is underlined. Potential N-linked glycosylation sites are shown in boldface.

Figure 3 shows an alignment of human and murine AGP-3, along with a consensus sequence (SEQ ID NO: 5). The predicted human and murine AGP-3 protein sequences were aligned by Fileup with gap creation penalty (12) and gap extension penalty (4) (Wisconsin GCG Package, Version 8.1, Genetics Computer Group Inc., Madison, Wisconsin). The consensus sequence was determined by Lineup (Wisconsin GCG Package, Version 8.1). The transmembrane regions from amino acid 47 to 72 in human AGP-3 and from amino acid 48 to 73 in murine AGP-3 are underlined. The N-terminal intracellular domain resides from amino acid 1 to 46 in human AGP-3 and from amino acid 1 to 47 in murine AGP-3. The C-terminal extracellular domain is localized from amino acid 73 to 285 in human AGP-3, and from amino acid 74 to 309. The human and murine AGP-3 share 68% amino acid identity overall. The C-terminus of AGP-3 is more conserved between human and mouse, with 87% identity over a 142-amino acid length. The putative conserved beta strands are indicated at the top, with the amino acids forming the putative strands underlined.

Figure 4 shows human and murine AGP-3 mRNA tissue distribution. Human tissue northern blots (A) and murine tissue northern blots (B) were probed with ^{32}P-labeled human AGP-3 probe (A) or murine AGP-3 probe. The probed blots were exposed to Kodak film for 18 hours (A) or seven days (B).

Figure 5 shows histology analysis of AGP-3 transgenic mouse spleen. The spleen sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and exosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The spleen of the transgenic mouse was enlarged, mainly due to the increase of size and number of the follicles. The B cell staining areas in the spleen follicles in the transgenic mouse were enlarged. The T cell number was slightly diminished.

Figure 6 shows histology analysis of AGP-3 transgenic mouse lymph nodes. The lymph node sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and exosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The lymph node size of the transgenic mouse was enlarged. The B cell number was greatly increased in the transgenic mouse. Instead of restricted to marginal zones of the follicles as in the control mouse, the B cells also filled out the follicular area in the lymph nodes of the transgenic mouse. The T cell number was decreased in the transgenic mouse as compared to the control.

Figure 7 shows histology analysis of AGP-3 transgenic mouse Peyer's patches. The Peyer's patches sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and exosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The histologic and immunohistologic changes were similar to the changes in the lymph node of the transgenic mouse.

Figure 8 shows FACS analysis of thymocytes, splenocytes and lymph node cells from AGP-3 transgenic mouse. Single-cell suspensions were prepared from spleen, lymph nodes and thymus from 10 AGP-3 transgenic mice and 5 control littermates. Cells were stained with FITC or PE-conjugated monoclonal antibodies against Thy-1.2, B220, CD11b, Gr-1,

CD4 or CD8. The B cell population increased by 100% in the transgenic mice as compared to the control mice. The T cell population decreased approximately 36%, with similar reductions in both CD4+ and CD8+ populations. Similar changes, though to a lesser degree, were observed in splenocytes. No differences in thymocyte staining were observed between the transgenic or control group.

Figure 9 shows a sequence comparison of the C-terminal region of members of the TNF ligand family determined via Pileup (Wisconsin GCG Package, Version 8.1). Amino acid numbers are indicated on the left side. The putative conserved beta strands and loops are indicated at the top. The predicted N-glycosylation sites are indicated with asterisks. The top line shows the consensus sequence (SEQ ID NO: 6). The remaining lines show the sequence for the C-terminal region of the mammalian TNF-related protein identified (SEQ ID NOS: 7 to 24, 40)

Figure 10 shows histology analysis of AGP-3 transgenic mice. Sections of spleen (A, B, C), lymph node (D, E, F) and Payer's patches (G, H, I) from control mice (left panel) and AGP-3 transgenic mice (right panel) were stained with hematoxylin and exosin (A, D, and G), anti-mouse B220 antibody (B, E, and H), or anti-mouse CD3 antibody (C, F, and I). Stained sections were analyzed under microscope at 10x.

Figure 11 shows FACS analysis splenocytes, lymph node cells and thymocytes of AGP-3 transgenic mice. Single-cell suspensions were prepared form spleen, lymph nodes and thymus from 10 AGP-3 transgenic mice and 5 control littermates. Cells with stained with FITC or PE-conjugated monoclonal antibodies against thy-1.2, B220, CD11b, Gr-1, CD4 or CD8.

Figure 12 shows elevation of serum immunoglobulin levels in AGP-3 transgenic mice. Control mice (n=5) and AGP-3 transgenic mice (n=5) were bled successively at 6, 7, 8, 9, 11 and 12 weeks of age. Serum IgM,

IgG, IgA, and IgE levels were quantitated by ELISA. Values are expressed as Mean ± SEM. All AGP-3 immunoglobulin levels were significantly increased (T-test; $P < 0.05$) compared to control groups.

Figure 13 shows kidney immunoglobulin deposits in AGP-3 transgenic mice. Kidney sections of 5 month control littermate (A, B, C), 5 month old AGP-3 mice (D, E, F), and 8 month old AGP-3 mice (G, H, I) were stained hematoxylin and exosin (A, D, and G), anti-mouse IgM (B, E, and H), anti-mouse IgG (C, F, and I), and Trichrome (G insert) Stained sections were analyzed under microscope at 60x.

Figure 14 shows that AGP-3 stimulates B cell survival and proliferation.

A. Increased B cell viability in AGP-3 transgenic mice. B cells were isolated from spleens of 3 month old AGP-3 transgenic mice (n-3) and control littermates (n=3). A total of 2.5×10^5 B cells was aliquoted per well in a 96-well round bottom plate and incubated for 9 days. At the indicated days, cells were incubated with 5 μg/ml Propidium Iodide and subject to FACS analysis for positive staining cells. Values are expressed as Mean ± SEM.

B. AGP-3 stimulates B cell proliferation. Purified B cells (10^5) from B6 mice were cultured in triplicates in 96 well plate with indicated amount of AGP-3 at the absence (upper panel) or presence of 2 μg/ml anti-IgM antibody (lower panel) for a period of 4 days. Proliferation was measured by radioactive ^{3}(H) thymidine uptake in last 18 hours of pulse. Data shown represent mean ± standard deviation of triplicate wells.

Detailed Description of the Invention

Definition of Terms

The following definitions apply to the terms used throughout this specification, unless otherwise limited in specific instances.

The term "AGP-3 related protein" refers to natural and recombinant proteins comprising the following sequence:

QDCLQLIADSXTPTIXKGXYTFVPWLLSF

(SEQ ID NO: 25)

wherein "X" may be any naturally occurring amino acid residue. This sequence is a consensus of the B and B' β-sheets and B/B' loop of hAGP-3 and mAGP-3 (see Figure 3), which is believed to be the specific receptor binding site. Preferred AGP-3-related proteins comprise both the B/B' consensus and the E/F consensus:

AMGHXIQRKKVHVFGDELSLVTLFR

(SEQ ID NO: 26)

The E/F region is also believed to be involved in receptor binding. More preferred proteins are those comprising the consensus of the B-I region:

QDCLQLIADS XTPTIXKGXY TFVPWLLSFK RGXALEEKEN KIXVXXTGYF FIYXQVLYTD XXXAMGHXIQ RKKVHVFGDE LSLVTLFRCI QNMPXTLPNN SCYSAGIAXL EEGDEXQLAI PRENAQISXX GDXTFFGALK LL

(SEQ ID NO: 27)

"AGP-3-related activity" means that a natural or recombinant protein, analog, derivative or fragment is capable of modulating B cell growth, survival, or activation, particularly in MLN, spleen, and Peyer's patches. The inventors contemplate that some molecules of interest may have activity antagonistic to native AGP-3 activity; for example, a derivative or analog may retain AGP-3 binding activity but will not activate the AGP-3 receptor. All such activity (agonism and antagonism of AGP-3) falls within the meaning of "AGP-3 related activity." Such activity can be determined, for example, by such assays as described in "Biological activity of AGP-3" in the Materials & Methods hereinafter, which may be modified as needed by many methods known to persons having ordinary skill in the art.

An "analog" of an AGP-3 protein (e.g., hAGP-3) is a polypeptide within the definition of "AGP-3-related protein" or "AGP-3-related protein," respectively, that has a substitution or addition of one or more amino acids. Such an AGP-3-related protein should maintain the property of eliciting B cell growth, survival, or activation. Such analogs will have substitutions or additions at any place along the polypeptide. Preferred analogs include those of soluble AGP-3-related proteins. Fragments or analogs may be naturally occurring, such as a polypeptide product of an allelic variant or a mRNA splice variant, or they may be constructed using techniques available to one skilled in the art for manipulating and synthesizing nucleic acids. The polypeptides may or may not have an amino terminal methionine residue.

A "derivative" of an AGP-3 protein is a polypeptide within the definition of "AGP-3-related protein" that has undergone post-translational modifications. Such modifications include, for example, addition of N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends, attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of an N-terminal methionine residue due to prokaryotic host cell expression. In particular, chemically modified derivatives of AGP-3-related protein that provide additional advantages such as increased stability, longer circulating time, or decreased immunogenicity are contemplated. Of particular use is modification with water soluble polymers, such as polyethylene glycol and derivatives thereof (see for example U.S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule,

or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties. Polypeptides may also be modified at pre-determined positions in the polypeptide, such as at the amino terminus, or at a selected lysine or arginine residue within the polypeptide. Other chemical modifications provided include a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

The term "protein" refers to polypeptides regardless of length or origin, comprising molecules that are recombinantly produced or naturally occurring, full length or truncated, having a natural sequence or mutated sequence, with or without post-translational modification, whether produced in mammalian cells, bacterial cells, or any other expression system.

The invention provides for proteins referred to as AGP-3 protein, or AGP-3-related proteins that primarily act on B cells. An EST bearing a portion of the AGP-3 sequence was obtained from a human fetal liver spleen cDNA library. A labeled cDNA fragment was used to probe a human spleen cDNA phage library (see "Cloning of Human AGP-3" in Materials & Methods hereinafter). The cDNA encoding a human AGP-3 was isolated from this phage library. The human protein is a type II transmembrane protein, having a short N-terminal intracellular region that differed from other members of the TNF ligand family and a long C-terminal extracellular region that comprises most of the conserved region of the TNF ligand family.

An EST encoding a murine ortholog was identified by BLAST search of Genebank using the human AGP-3 sequence. The corresponding cDNA clone was obtained from a mouse lymph node library and used to probe a mouse spleen cDNA phage library (see Materials & Methods

hereinafter). The cDNA encoding a murine AGP-3 ortholog was isolated from this phage library.

Northern blots were used to determine tissue distribution of transcription of AGP-3 (see "Cloning of Murine AGP-3" in Materials & Methods hereinafter). In murine tissue, AGP-3 mRNA was detected mainly in spleen, lung, liver, and kidney. In human tissue, AGP-3 mRNA was detected predominantly in peripheral blood leukocytes, with weaker transcription in spleen, lung, and small intestine (see Figures 4A and 4B).

The murine ortholog of AGP-3 was overexpressed in transgenic mice (see "Overexpression of murine AGP-3 in transgenic mice" in Materials & Methods hereinafter). In these transgenic mice, serum globulin and total protein levels increased greatly over control littermates while the albumin level remained the same (see "Biological Activity of AGP-3" in Materials & Methods hereinafter). The mice also exhibited increases in the size and number of follicles in the spleen, lymph nodes, and Peyer's patches (Figures 5, 6, and 7). In their MLN, the mice exhibited 100% increases in the number of cells expressing CD45 receptor with concomitant decreases in cells expressing CD90, CD4, and CD8. These results correspond to an increase in the B cell population and a decrease in the T cell population in the MLN (Figures 6 and 8). Similar results were obtained in the spleen, but to a lesser extent (Figures 5 and 8).

Nucleic Acids

The invention provides for isolated nucleic acids encoding AGP-3-related proteins. As used herein, the term "nucleic acid" comprises cDNA, genomic DNA, wholly or partially synthetic DNA, and RNA. These nucleic acids may be prepared or isolated as described in the working examples hereinafter or by nucleic acid hybridization thereof.

Nucleic acid hybridization typically involves a multi-step process. A first hybridization step forms nucleic acid duplexes from single strands.

A second hybridization step under more stringent conditions selectively retains nucleic acid duplexes having the desired homology. The conditions of the first hybridization step are generally not crucial, provided they are not of higher stringency than the second hybridization step. Generally, the second hybridization is carried out under conditions of high stringency, wherein "high stringency" conditions refers to conditions of temperature and salt that are about 12-20 °C below the melting temperature (T_m) of a perfect hybrid of part or all of the complementary strands corresponding to Figure 1 (SEQ ID NO: 1) and Figure 2 (SEQ ID NO: 3). In one embodiment, "high stringency" conditions refer to conditions of about 65 °C and not more than about 1 M Na^+. It is understood that salt concentration, temperature and/or length of incubation may be varied in either the first or second hybridization steps such that one obtains the hybridizing nucleic acid molecules according to the invention. Conditions for hybridization of nucleic acids and calculations of T_m for nucleic acid hybrids are described in Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, New York.

The nucleic acids of the invention may hybridize to part or all of the polypeptide coding regions of AGP-3 related proteins (e.g., SEQ ID NOS: 2 and 4 as shown in Figures 1 and 2) and therefore may be truncations or extensions of the nucleic acid sequences shown therein. Truncated or extended nucleic acids are encompassed by the invention provided that the encoded proteins retain AGP-3 related activity. In one embodiment, the nucleic acid will encode a polypeptide of at least about 10 amino acids. In another embodiment, the nucleic acid will encode a polypeptide of at least about 20 amino acids. In yet another embodiment, the nucleic acid will encode a polypeptide of at least about 50 amino acids. The hybridizing nucleic acids may also include noncoding sequences located 5' and/or 3' to the coding regions for the AGP-3 related protein. Noncoding

sequences include regulatory regions involved in expression of AGP-3 related protein, such as promoters, enhancer regions, translational initiation sites, transcription termination sites and the like.

In preferred embodiments, the nucleic acids of the invention encode mouse or human AGP-3. Most preferred are the nucleic acids encoding proteins of SEQ ID NOS: 25, 26, or 27. Nucleic acids may encode a membrane-bound form of AGP-3-related protein or soluble forms. For human AGP-3-related protein, the predicted transmembrane region includes amino acid residues 47-72 inclusive as shown in Figure 1 (SEQ. ID. NO: 2); for murine AGP-3 related protein, residues 48-73 inclusive as shown in Figure 2 (SEQ ID NO: 4). Substitutions that replace hydrophobic amino acid residues in this region with neutral or hydrophilic amino acid residues would be expected to disrupt membrane association and result in soluble AGP-3-related protein. In addition, deletions of part or all the transmembrane region would also be expected to produce soluble forms of AGP-3-related protein. Nucleic acids encoding SEQ ID NO: 5 as shown in Figure 3 or fragments and analogs thereof, encompass soluble AGP-3-related proteins.

Nucleic acid sequences of the invention may also be used for the detection of sequences encoding AGP-3-related protein in biological samples. In particular, the sequences may be used to screen cDNA and genomic libraries for related AGP-3-related protein sequences, especially those from other species. The nucleic acids are also useful for modulating levels of AGP-3-related protein by anti-sense technology or in vivo gene expression. Development of transgenic animals expressing AGP-3-related protein are useful for production of the polypeptides and for the study of in vivo biological activity.

Vectors and Host Cells

The nucleic acids of the invention will be linked with DNA sequences so as to express biologically active AGP-3-related protein. Sequences required for expression are known to those skilled in the art and include promoters and enhancer sequences for initiation of RNA synthesis, transcription termination sites, ribosome binding sites for the initiation of protein synthesis, and leader sequences for secretion. Sequences directing expression and secretion of AGP-3-related protein may be homologous, i.e., the sequences are identical or similar to those sequences in the genome involved in AGP-3-related protein expression and secretion, or they may be heterologous. A variety of plasmid vectors are available for expressing AGP-3-related protein in host cells (see, for example, Methods in Enzymology v. 185, Goeddel, D.V. ed., Academic Press (1990)). For expression in mammalian host cells, a preferred embodiment is plasmid pDSRα described in PCT Application No. 90/14363. For expression in bacterial host cells, preferred embodiments include plasmids harboring the lux promoter (see co-owned and co-pending U.S. Serial No. 08/577,778, filed December 22, 1995). In addition, vectors are available for the tissue-specific expression of AGP-3-related protein in transgenic animals. Gene transfer vectors derived from retrovirus (RV), adenovirus (AdV), and adeno-associated virus (AAV) may also be used for the expression of AGP-3 related protein in human cells for in vivo therapy (see PCT Application No. 86/00922).

Prokaryotic and eukaryotic host cells expressing AGP-3-related protein are also provided by the invention. Host cells include bacterial, yeast, plant, insect or mammalian cells. AGP-3-related protein may also be produced in transgenic animals, such as mice or goats. Plasmids and vectors containing the nucleic acids of the invention are introduced into appropriate host cells using transfection or transformation techniques known to one skilled in the art. Host cells may contain DNA sequences

encoding AGP-3-related protein as shown in Figures 1, 2, or 3, or a portion of either thereof, such as the extracellular domain or the cytoplasmic domain. Nucleic acids encoding AGP-3-related proteins may be modified by substitution of codons that allow for optimal expression in a given host. At least some of the codons may be so-called preference codons that do not alter the amino acid sequence and are frequently found in genes that are highly expressed. However, it is understood that codon alterations to optimize expression are not restricted to the introduction of preference codons. Examples of preferred mammalian host cells for AGP-3-related protein expression include, but are not limited to COS, CHOd-, 293 and 3T3 cells. A preferred bacterial host cell is Escherichia coli.

Polypeptides

The invention also provides AGP-3-related proteins as the products of prokaryotic or eukaryotic expression of exogenous DNA sequences. Exogenous DNA sequences include cDNA, genomic DNA and synthetic DNA sequences. AGP-3-related proteins may be the products of bacterial, yeast, plant, insect or mammalian cells expression, or from cell-free translation systems. AGP-3-related proteins produced in bacterial cells will have N-terminal methionine residues. The invention also provides for a process of producing AGP-3-related proteins comprising growing prokaryotic or eukaryotic host cells transformed or transfected with nucleic acids encoding them and isolating polypeptide expression products of the nucleic acids.

Polypeptides that are mammalian proteins or are fragments, analogs or derivatives thereof are encompassed by the invention. In preferred embodiments, the AGP-3-related protein is human AGP-3 protein. A fragment of AGP-3-related protein refers to a polypeptide having a deletion of one or more amino acids such that the resulting polypeptide retains AGP-3 related activity; for example, the polypeptide

has at least the property of eliciting or antagonizing B cell growth, survival, or activation, especially in mesenteric lymph nodes. Said fragments will have deletions originating from the amino terminal end, the carboxy terminal end, and internal regions of the polypeptide. Fragments of AGP-3-related proteins are at least about ten amino acids, at least about 20 amino acids, or at least about 50 amino acids in length. In preferred embodiments, AGP-3-related proteins will have a deletion of one or more amino acids from the transmembrane region (amino acid residues 48-73 as shown in Figure 1), or, alternatively, one or more amino acids from the amino-terminus up to and/or including the transmembrane region (amino acid residues 1-73 as shown in Figure 1). Such polypeptides may act as agonists or antagonists of the ligand:receptor interaction and activate or inhibit ligand-mediated activity of AGP-3 related protein. Such antagonists and/or agonists can be examined for AGP-3 related activity (see "Biological activity of AGP-3" in Materials & Methods hereinafter).

The polypeptides of the invention are isolated and purified from tissues and cell lines that express AGP-3 related protein, either extracted from lysates or from conditioned growth medium, and from transformed host cells expressing AGP-3 related protein. Human AGP-3 related protein, or nucleic acids encoding same, may be isolated from human lymph node or fetal liver tissue. Isolated AGP-3 related protein is free from association with human proteins and other cell constituents.

A method for purification of such proteins from natural sources (e.g. tissues and cell lines that normally express an AGP-3 related protein) and from transfected host cells is also encompassed by the invention. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The chromatography steps can include ion exchange, gel filtration,

hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-AGP-3-related protein antibody or biotin-streptavidin affinity complex and the like.

Fusion proteins and derivatives

The invention further comprises AGP-3-related protein chimeras, as well as such proteins derivatized by linkage to such molecules as PEG or dextran. Such proteins comprise part or all of an AGP-3-related protein amino acid sequence fused to a heterologous amino acid sequence. The heterologous sequence may be any sequence that allows the resulting fusion protein to retain AGP-3-related activity (i.e., AGP-3 agonists) or will maintain AGP-3 binding activity but not have AGP-3 related activity as defined herein (i.e., AGP-3 antagonists). Such fragments, derivatives or analogs of AGP-3 can be examined for their ability to agonize or antagonize AGP-3-mediated B cell growth, survival, or activation associated with the disease or condition (see "Biological activity of AGP-3" in Materials & Methods hereinafter). In preferred embodiments, a heterologous sequence is fused to a sequence comprising an AGP-3 related protein's B/B' region (SEQ ID NO: 25) and/or the E/F region (SEQ ID NO: 26) or to the more complete B-I region (SEQ ID NO: 27). Such heterologous sequences include cytoplasmic domains that allow for alternative intracellular signaling events, sequences that promote oligomerization (e.g., the Fc region of IgG), enzyme sequences that provide a label for the polypeptide, and sequences that provide affinity probes (e.g., an antigen-antibody recognition site).

Preferred molecules in accordance with this invention are Fc-linked AGP-3 related proteins. Useful modifications of protein therapeutic agents by fusion with the "Fc" domain of an antibody are discussed in detail in a patent application entitled, "Modified Peptides as Therapeutic Agents," U.S. Ser. No. 09/428,082, PCT appl. no. WO 99/25044, which is hereby

incorporated by reference in its entirety. That patent application discusses linkage to a "vehicle" such as PEG, dextran, or an Fc region.

In the compositions of matter prepared in accordance with this invention, the AGP-3 related protein may be attached to a vehicle through the protein's N-terminus or C-terminus. Thus, the vehicle-protein molecules of this invention may be described by the following formula I:

I

$$(X^1)_a\text{-}F^1\text{-}(X^2)_b$$

wherein:

F^1 is a vehicle (preferably an Fc domain);

X^1 and X^2 are each independently selected from $-(L^1)_c\text{-}P^1$, $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2$, $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3$, and $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3\text{-}(L^4)_f\text{-}P^4$

P^1, P^2, P^3, and P^4 are each independently sequences of AGP-3 related protein (e.g., a fragment of hAGP-3);

L^1, L^2, L^3, and L^4 are each independently linkers; and

a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1.

Thus, compound I comprises preferred compounds of the formulae

II

$$X^1\text{-}F^1$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the C-terminus of X^1;

III

$$F^1\text{-}X^2$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the N-terminus of X^2;

IV

$$F^1\text{-}(L^1)_c\text{-}P^1$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the N-terminus of $-(L^1)_c-P^1$; and

V

$$F^1-(L^1)_c-P^1-(L^2)_d-P^2$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the N-terminus of $-L^1-P^1-L^2-P^2$.

Antibodies

Uses for antibodies specifically binding the polypeptides of the invention are also encompassed by the invention. The antibodies may be generated by immunization with full-length AGP-3 related protein, or fragments thereof. Preferred antibodies bind to SEQ ID NOS: 25, 26, or 27. Such antibodies may be generated by immunization with polypeptides comprising those sequences. The term "antibodies" also refers to molecules having Fv, Fc and other structural domains usually associated with antibodies but that may be generated by other techniques (e.g., phage display antibody generation). The antibodies of the invention may be polyclonal or monoclonal, or may be recombinant antibodies, such as chimeric antibodies wherein the murine constant regions on light and heavy chains are replaced by human sequences, or CDR-grafted antibodies wherein only the complementarity determining regions are of murine origin. Antibodies of the invention may also be fully human antibodies prepared, for example, by immunization of transgenic animals capable of producing human antibodies (see, for example, PCT Application No. WO93/12227). Regardless of the means by which they are generated, antibodies in accordance with this invention may be produced by recombinant means (e.g., transfection of CHO cells with vectors comprising antibody sequence).

The antibodies are useful for detecting AGP-3 related protein in biological samples, thereby allowing the identification of cells or tissues

that produce such proteins. In addition, antibodies that bind to AGP-3 related proteins and block interaction with other binding compounds (i.e., "antagonist antibodies") have therapeutic use in modulating B cell growth, activation, and/or proliferation. Antibodies can be tested for binding to AGP-3 related protein and examined for their effect on AGP-3-mediated B cell growth, survival, or activation associated with the disease or condition (see "Biological activity of AGP-3" in Materials & Methods hereinafter).

Compositions

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the AGP-3 related protein of the invention together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of an AGP-3 related protein agonist or antagonist. The term "therapeutically effective amount" means an amount that provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form and comprises one or more of the following:

- a diluent (e.g., Tris, acetate or phosphate buffers) having various pH values and ionic strengths;
- a solubilizer (e.g., Tween or Polysorbate);
- carriers (e.g., human serum albumin or gelatin);
- preservatives (e.g., thimerosal or benzyl alcohol); and
- antioxidants (e.g., ascorbic acid or sodium metabisulfite).

Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive survey of component suitable for pharmaceutical compositions is found in

Remington's Pharmaceutical Sciences (1980), 18th ed. (A. R. Gennaro, ed.) Mack, Easton, PA.

In a preferred embodiment, compositions comprising AGP-3 antibody or soluble AGP-3-related protein are provided. Also encompassed are compositions comprising soluble AGP-3-related protein modified with water-soluble polymers to increase solubility, stability, plasma half-life and bioavailability. Compositions may also comprise incorporation of soluble AGP-3 related protein into liposomes, microemulsions, micelles or vesicles for controlled delivery over an extended period of time. Soluble AGP-3 related protein may be formulated into microparticles suitable for pulmonary administration.

Compositions of the invention may be administered by injection (either subcutaneous, intravenous or intramuscular) or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one of ordinary skill in the art.

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the coding region of AGP-3 related protein and/or flanking regions to cells and tissues as part of an anti-sense therapy regimen.

Pharmaceutical Methods of Use

AGP-3 related proteins and agonists or antagonists thereof may be used to treat conditions characterized by B cell growth, survival, and activation, such as autoimmune and inflammatory disorders. The invention also encompasses modulators (agonists and antagonists) of AGP-3-related protein and methods for obtaining them. Such a modulator may either increase or decrease at least one activity associated with AGP-

3, such as B cell growth, survival, or activation in MLN, spleen, and Peyer's patches. Typically, an agonist or antagonist may be a co-factor, such as a protein, peptide, carbohydrate, lipid or small molecular weight molecule, that interacts with AGP-3 and regulates activity. Potential polypeptide antagonists include antibodies that react with soluble or membrane-associated forms of AGP-3, a fragment of AGP-3 (e.g., SEQ ID NO: 25) and an Fc-linked AGP-3 fragment. Molecules that regulate AGP-3-related protein expression typically include nucleic acids that are complementary to nucleic acids encoding AGP-3-related protein and that act as anti-sense regulators of expression.

AGP-3-related proteins and modulators thereof may be particularly useful in treatment of inflammatory conditions of the joints. Inflammatory conditions of a joint are chronic joint diseases that afflict and disable, to varying degrees, millions of people worldwide. Rheumatoid arthritis is a disease of articular joints in which the cartilage and bone are slowly eroded away by a proliferative, invasive connective tissue called pannus, which is derived from the synovial membrane. The disease may involve peri-articular structures such as bursae, tendon sheaths and tendons as well as extra-articular tissues such as the subcutis, cardiovascular system, lungs, spleen, lymph nodes, skeletal muscles, nervous system (central and peripheral) and eyes (Silberberg (1985), Anderson's Pathology, Kissane (ed.), II:1828). Osteoarthritis is a common joint disease characterized by degenerative changes in articular cartilage and reactive proliferation of bone and cartilage around the joint. Osteoarthritis is a cell-mediated active process that may result from the inappropriate response of chondrocytes to catabolic and anabolic stimuli. Changes in some matrix molecules of articular cartilage reportedly occur in early osteoarthritis (Thonar et al. (1993), Rheumatic disease clinics of North America, Moskowitz (ed.), 19:635-657 and Shinmei et al. (1992), Arthritis Rheum.,

35:1304-1308). AGP-3, AGP-3 R and modulators thereof are believed to be useful in the treatment of these and related conditions.

AGP-3 related proteins and agonists or antagonists thereof may also be useful in treatment of a number of additional diseases and disorders, including:

acute pancreatitis;

ALS;

Alzheimer's disease;

asthma;

atherosclerosis;

cachexia/anorexia;

chronic fatigue syndrome;

diabetes (e.g., insulin diabetes);

fever;

glomerulonephritis;

graft versus host disease;

hemorrhagic shock;

hyperalgesia;

inflammatory bowel disease;

inflammatory conditions of a joint, including osteoarthritis, psoriatic arthritis and rheumatoid arthritis;

inflammatory conditions resulting from strain, sprain, cartilage damage, trauma, orthopedic surgery, infection or other disease processes;

ischemic injury, including cerebral ischemia (e.g., brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration);

learning impairment;

lung diseases (e.g., ARDS);

multiple myeloma;

multiple sclerosis;

myelogenous leukemia (e.g., AML and CML) and other leukemias;

myopathies (e.g., muscle protein metabolism, esp. in sepsis);

neurotoxicity (e.g., as induced by HIV);

osteoporosis;

pain;

Parkinson's disease;

pre-term labor;

psoriasis;

reperfusion injury;

septic shock;

side effects from radiation therapy;

sleep disturbance;

temporal mandibular joint disease; and

tumor metastasis.

Agonists and antagonists of AGP-3-related protein may be administered alone or in combination with a therapeutically effective amount of other drugs, including analgesic agents, disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and any immune and/or inflammatory modulators. Thus, agonists and antagonists of AGP-3 related protein may be administered with:

- Modulators of other members of the TNF/TNF receptor family, including TNF antagonists, such as etanercept (Enbrel™), sTNF-RI, D2E7, and Remicade™.
- Nerve growth factor (NGF) modulators.
- IL-1 inhibitors, including IL-1ra molecules such as anakinra (Kineret™) and more recently discovered IL-1ra-like molecules such as IL-1Hy1 and IL-1Hy2; IL-1 "trap" molecules as described

in U.S. Pat. No. 5,844,099, issued December 1, 1998; IL-1 antibodies; solubilized IL-1 receptor, and the like.

- IL-6 inhibitors (e.g., antibodies to IL-6).
- IL-8 inhibitors (e.g., antibodies to IL-8).
- IL-18 inhibitors (e.g., IL-18 binding protein, solubilized IL-18 receptor, or IL-18 antibodies).
- Interleukin-1 converting enzyme (ICE) modulators.
- insulin-like growth factors (IGF-1, IGF-2) and modulators thereof.
- Transforming growth factor-β (TGF-β), TGF-β family members, and TGF-β modulators.
- Fibroblast growth factors FGF-1 to FGF-10, and FGF modulators.
- Osteoprotegerin (OPG), OPG analogues, osteoprotective agents, and bone anabolic agents.
- PAF antagonists.
- Keratinocyte growth factor (KGF), KGF-related molecules (e.g., KGF-2), and KGF modulators.
- COX-2 inhibitors, such as Celebrex™ and Vioxx™.
- Prostaglandin analogs (e.g., E series prostaglandins).
- Matrix metalloproteinase (MMP) modulators.
- Nitric oxide synthase (NOS) modulators, including modulators of inducible NOS.
- Modulators of glucocorticoid receptor.
- Modulators of glutamate receptor.
- Modulators of lipopolysaccharide (LPS) levels.
- Anti-cancer agents, including inhibitors of oncogenes (e.g., fos, jun) and interferons.
- Noradrenaline and modulators and mimetics thereof.

Assay Methods of Use

AGP-3-related proteins may be used in a variety of assays for detecting agonists, antagonists and characterizing interactions with AGP-3 related proteins. In general, the assay comprises incubating AGP-3-related protein under conditions that permit measurement of AGP-3-related activity as defined above. Qualitative or quantitative assays may be developed. Assays may also be used to identify new AGP-3 agonists or antagonists and AGP-3 related potein family members.

Binding assays for agonists, or antagonists to natural or recombinant AGP-3 related protein may be carried out in several formats, including cell-based binding assays, membrane binding assays, solution-phase assays and immunoassays. In general, trace levels of a labeled binding molecule are incubated with AGP-3-related protein samples for a specified period of time followed by measurement of bound molecule by filtration, electrochemiluminescent (ECL, ORIGEN system by IGEN), cell-based or immunoassays. Homogeneous assay technologies for radioactivity (SPA; Amersham) and time-resolved fluoresence (HTRF, Packard) can also be implemented. Binding is detected by labeling a binding molecule (e.g., an anti-AGP-3 antibody) with radioactive isotopes (125I, 35S, 3H), fluorescent dyes (fluorescein), lanthanide (Eu^{3+}) chelates or cryptates, orbipyridyl-ruthenium (Ru^{2+}) complexes. It is understood that the choice of a labeled probe will depend upon the detection system used. Alternatively, a binding molecule may be modified with an unlabeled epitope tag (e.g., biotin, peptides, His_6, myc) and bound to proteins such as streptavidin, anti-peptide or anti-protein antibodies that have a detectable label as described above.

Binding molecules in such assays may be nucleic acids, proteins, peptides, carbohydrates, lipids or small molecular weight organic compounds. The binding molecule may be substantially purified or

present in a crude mixture. The binding molecules may be further characterized by their ability to increase or decrease AGP-3 related activity in order to determine whether they act as an agonist or an antagonist.

In an alternative method, AGP-3-related protein may be assayed directly using polyclonal or monoclonal antibodies to AGP-3 related proteins in an immunoassay. Additional forms of AGP-3-related proteins containing epitope tags as described above may be used in solution and immunoassays.

AGP-3 related proteins are also useful for identification of intracellular proteins that interact with their respective cytoplasmic domains by a yeast two-hybrid screening process. As an example, hybrid constructs comprising DNA encoding the N-terminal 50 amino acids of an AGP-3 related protein fused to a yeast GAL4-DNA binding domain may be used as a two-hybrid bait plasmid. Positive clones emerging from the screening may be characterized further to identify interacting proteins. This information may help elucidate an intracellular signaling mechanism associated with AGP-3-related activity and provide intracellular targets for new drugs that modulate inflammatory and immune-related diseases and conditions.

A variety of assays may be used to measure the interaction of AGP-3-related proteins and agonists, antagonists, or other ligands in vitro using purified proteins. These assays may be used to screen compounds for their ability to increase or decrease the rate or extent of binding to AGP-3 related proteins. In one type of assay, AGP-3 related protein can be immobilized by attachment to the bottom of the wells of a microtiter plate. A radiolabeled binding molecule and a test molecule can then be added either one at a time (in either order) or simultaneously to the wells. After incubation, the wells can be washed and counted using a scintillation counter for radioactivity to determine the extent of binding to AGP-3

related protein. Typically, molecules will be tested over a range of concentrations, and a series of control wells lacking one or more elements of the test assays can be used for accuracy in evaluation of the results. An alternative to this method involves reversing the "positions" of the proteins; i.e., immobilizing a binding molecule to the mictrotiter plate wells, incubating with the test compound and radiolabeled AGP-3 related protein, and determining the extent of binding. See, for example, chapter 18 of Current Protocols in Molecular Biology (1995) (Ausubel et al., eds.), John Wiley & Sons, New York, NY.

As an alternative to radiolabeling, AGP-3 related proteins or a binding molecule may be conjugated to biotin and the presence of biotinylated protein can then be detected using streptavidin linked to an enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), that can be detected colorimetrically, or by fluorescent tagging of streptavidin. An antibody directed to AGP-3 related protein or a binding molecule that is conjugated to biotin may also be used and can be detected after incubation with enzyme-linked streptavidin linked to AP or HRP.

AGP-3-related proteins or binding molecules may also be immobilized by attachment to agarose beads, acrylic beads or other types of such inert substrates. The substrate-protein complex can be placed in a solution containing the complementary protein and the test compound; after incubation, the beads can be precipitated by centrifugation, and the amount of binding between the AGP-3-related protein and a binding molecule can be assessed using the methods described above. Alternatively, the substrate-protein complex can be immobilized in a column and the test molecule and complementary molecule passed over the column. Formation of a complex between AGP-3 related protein and the binding molecule can then be assessed using any of the techniques set forth above (i.e., radiolabeling, antibody binding, and the like).

Another useful in vitro assay is a surface plasmon resonance detector system, such as the Biacore assay system (Pharmacia, Piscataway, NJ). The Biacore system may be carried out using the manufacturer's protocol. This assay essentially involves covalent binding of either an AGP-3 related protein or a binding molecule to a dextran-coated sensor chip that is located in a detector. The test compound and the other complementary protein can then be injected into the chamber containing the sensor chip either simultaneously or sequentially and the amount of complementary protein that binds can be assessed based on the change in molecular mass that is physically associated with the dextran-coated side of the of the sensor chip; the change in molecular mass can be measured by the detector system.

In vitro assays such as those described above may be used advantageously to screen rapidly large numbers of compounds for effects on complex formation with AGP-3-related proteins. The assays may be automated to screen compounds generated in phage display, synthetic peptide and chemical synthesis libraries.

Compounds that increase or decrease complex formation of AGP-3-related proteins and binding molecules may also be screened in cell culture using cells and cell lines bearing such ligands. Cells and cell lines may be obtained from any mammal, but preferably will be from human or other primate, canine, or rodent sources. Such cells may be enriched from other cell types by affinity chromatography using publicly available procedures. Attachment of AGP-3-related protein to such cells is evaluated in the presence or absence of test compounds and the extent of binding may be determined by, for example, flow cytometry using a biotinylated antibody. Cell culture assays may be used advantageously to further evaluate compounds that score positive in protein binding assays described above.

Description of Preferred Embodiments

The following examples are offered to illustrate the invention, but should not be construed as limiting the scope thereof.

Materials and Methods

Cloning of Human AGP-3

A TNF family profile search of the Genbank dbEST data base was performed. Smith et al.(1994), Cell, 76: 959-62; Luethy et al.(1994), Protein Science, 3: 139-46. One human EST sequence (GenBank accession number T87299) was identified as a possible new member of the TNF ligand. The EST was obtained from human fetal liver spleen cDNA library (The WashU-Merck EST Project). The cDNA clone (115371 3') corresponding to the EST sequence was obtained from Genome Systems, Inc. (St. Louis, MO). The cDNA fragment was released from the pT7T3D vector with EcoRI and NotI digestion. The fragment was approximately 0.7 kb in length and was used for the subsequent full-length cloning.

The ^{32}P-dCTP-labeled T87299 cDNA fragment was used as a probe to screen a human spleen cDNA phage library (Stratagene, La Jolla, CA). Recombinant phages were plated onto E. coli strain XL1-blue at approximately 5 x 10^4 transformants per 150 mm LB plate. Nitrocellulose filters were lifted from these plates in duplicates. Filters were prehybridized in 5x SSC, 50% deionized formamide, 5x Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA for 2 hours at 42 °C. The filters were then hybridized in the same solution with the addition of 5 ng/ml of labeled probe at 42°C overnight. The filters were first washed in 2x SSC and 0.1% SDS for 10 minutes at RT twice, and then washed in 0.1x SSC and 0.1% SDS at 65 °C for 30 minutes twice. The filters were then exposed to autoradiography with intensifying screens at -80 °C overnight. Positive hybridizing plaques were determined by aligning the duplicate filters, and then picked up for subsequent

secondary or tertiary screening till single isolated positive plaque was obtained. From total of one million recombinant phage clones, 8 positive plaques were obtained.

The pBluescript phagemid was excised from phage using the ExAssist™/SOLR™ System according to the manufacturer's description (Stratagene, La Jolla, CA). The excised phagemids were plated onto freshly grown SOLR cells on LB/ampicillin plates and incubated overnight. Single bacteria colony was amplified in LB media containing 100 μg/ml ampicillin. The plasmid DNA was prepared and both strands of cDNA insert were sequenced.

The human AGP-3 cDNA (clone 13-2) is 1.1 kb in length. It encodes a LORF of 285 amino acids. FASTA search of the SwissProt database with the predicted AGP-3 protein sequence indicated that it is mostly related to human TNFα with 25% identity in C-terminal 116 amino acid overlap. Like other TNF ligand family members, human AGP-3 protein is a type II transmembrane protein, containing a short N-terminal intracellular domain (amino acids 1-46), a hydrophobic transmembrane region (amino acids 47-68) following by a long C-terminal extracellular domain (amino acid 69-285). The C-terminal extracellular domain of AGP-3 contained most of the conserved region of the TNF ligand family. Smith et al.(1994), Cell, 76: 959-62.

Cloning of Murine AGP-3

An EST sequence (Genebank accession number AA254047) encoding a potential murine AGP-3 ortholog was identified by BLAST search of Genebank dbEST database with human AGP-3 sequence. The corresponding cDNA clone (722549 5') from mouse lymph node library was obtained from Genome Systems, Inc. (St. Louis, MO). The clone contained a 0.9 kb cDNA insert which could be released by EcoRI and NotI digestion. The 0.9 kb cDNA fragment encodes an open reading frame

of 96 amino acids which shares 87% identity with the corresponding C-terminal human AGP-3 polypeptide sequence. A 0.41 kb EcoRI-XmnI fragment, which contained 290 bp coding region and 120 bp 3' non-coding region, was used as probe to screening a mouse spleen cDNA phage library (Stratagene, La Jolla, CA) for full length murine AGP-3 cDNA as described above. From one million recombinant phage clones, 6 positive plaques were obtained. The phagemid was excised from phage as described above. The plasmid DNA was prepared and both strands of cDNA insert were sequenced. The murine AGP-3 cDNA (clone S6) encodes a polypeptide of 309 amino acids. Like its human ortholog, murine AGP-3 is also a type II transmembrane region, containing a short N-terminal intracellular domain (amino acid 1-46), a hydrophobic transmembrane region (amino acid 47-68) following by a long C-terminal extracellular domain (amino acid 69-285). The human and murine AGP-3 share 68% amino acid sequence identity overall. However, the C-terminal 142 amino acid sequences share 87% identity between the two species. Preceding the highly conserved C-terminus region, there is an insertion of 30 extra amino acids in the murine AGP-3. Four out of 7 positive phage plaques were independent clones, yet they all shared the same coding sequences.

Expression of human and murine AGP-3 mRNA

Multiple human or murine tissue northern blots (Clontech, Palo Alto, CA) were probed with ^{32}P-dCTP labeled human AGP-3 0.7kb EcoRI-NotI fragment or murine AGP-3 0.41kb EcoRI-XmnI fragment, respectively. The Northern blots were prehybridized in 5x SSC, 50% deionized formamide, 5xDenhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA for 2 hours at 42 °C. The blots were then hybridized in the same solution with the addition of 5ng/ml of labeled probe at 42°C overnight. The filters were first washed in 2x SSC and 0.1%

SDS for 10 minutes at RT twice, and then washed in 0.1x SSC and 0.1% SDS at 65 °C for 30 minutes twice. The blots were then exposed to autoradiography. The human tissue northern blot analysis with human AGP-3 probe under stringent conditions revealed predominant AGP-3 transcripts with a related molecular mass of 2.4kb in peripheral blood leukocytes (Figure 4A). Weaker expression was also detected in human spleen, lung and small intestine (Figure 4A). Among murine tissues analyzed, murine AGP-3 mRNA, with a relative molecular mass of 2kb, was mainly detected in spleen, lung, liver and kidney (Figure 4B).

Overexpression of murine AGP-3 in transgenic mice

Murine AGP-3 cDNA clone S6 in pBluescript SK(-) in pBluescript was used as template to PCR the entire coding region. T3 primer

5′ AAT TAA CCC TCA CTA AAG GG 3″

SEQ ID NO: 28

was used as 5′ PCR primer. The 3′ end PCR primer, which contained a XhoI site, was

5′ TCT CCC TCG AGA TCA CGC ACT CCA GCA AGT GAG 3′

SEQ ID NO: 29

PCR reactions were carried in a volume of 50 µl with 1 unit of vent DNA polymerase (New England Biolabs) in 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.1% Triton-X100, 10 µM of each dNTP, 1 µM of each primer and 10 ng of murine AGP-3 cDNA template. Reactions were performed in 94 °C for 45 s, 55 °C for 55 S, and 72 °C for 2 minutes, for a total of 35 cycles. The PCR fragment created a XhoI site at 3′ end after the AGP-3 coding region. The 1 kb PCR fragment was purified by electrophoresis, and digested with XbaI (present in the pBluescript MCS, 80 bp upstream of AGP-3 starting Methione) and XhoI restriction enzymes. The XbaI-XhoI PCR fragment was cloned into expression vector under the control of the human β-actin promoter. Graham et al.(1997), Nature Genetics 17: 272-4;

Ray et al.(1991), Genes Dev. 5: 2265-73. The PCR fragment was sequenced to ensure no mutation. The murine AGP-3 expression plasmid was purified through two rounds of CsCl density gradient centrifugation. The purified plasmid was digested with ClaI, and a 6 kb fragment containing murine AGP-3 transgene was purified by gel electrophoresis. The purified fragment was resuspended in 5 mM Tris, pH 7.4, 0.2 mM EDTA at 2 µg/ml concentration. Single-cell embryos from BDF1 x BDF1-bred mice were injected as described (WO97 /23614). Embryos were cultured overnight in a CO_2 incubator and 15-20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

Following term pregnancy, 62 offspring were obtained from implantation of microinjected embryos. The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. Ear pieces were digested in 20 µl ear buffer (20mM Tris, pH8.0, 10mM EDTA, 0.5% SDS, 500 µg/ml proteinase K) at 55°C overnight. The sample was diluted with 200 µl of TE, and 2 µl of the ear sample was used for the PCR reaction. The 5′ PCR primer

5′ AAC AGG CTA TTT CTT CAT CTA CAG 3′

SEQ ID NO: 30

resided in the murine AGP-3 coding region. The 3′ PCR primer

5′ CTC ATC AAT GTA TCT TAT CAT GTC T 3′

SEQ ID NO: 31

resided in the vector 3′ to the murine AGP-3 transgene. The PCR reactions were carried in a volume of 50 µl with 0.5 unit of Tag DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 10 µM of each dNTP, 1 µM of each primer and 2 µl of ear sample. The mixtures were first heated at 94 °C for 2 min, and the PCR reactions were performed in 94 °C for 30 s, 55 °C for 30 s, and 72 °C

for 45 s, for a total of 35 cycles. Of the 62 offspring, 10 were identified as PCR positive transgenic founders.

At 8 weeks of age, all ten transgenic founders (animal 3, 6, 9, 10, 13, 38, 40, 58, 59, and 62) and five controls (animal 7, 8, 11, 12 and 14) were sacrificed for necropsy and pathological analysis. Portions of spleen were removed, and total cellular RNA was isolated from the spleens of all the transgenic founders and negative controls using the Total RNA Extraction Kit (Qiagen Inc., Chartsworth, CA). The expression of the transgene was determined by RT-PCR. The cDNA was synthesized using the SuperScript™ Preamplification System according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD). The primer

5′ CTC ATC AAT GTA TCT TAT CAT GTC T 3′

SEQ ID NO: 32

which was located in the expression vector sequence 3′ to the AGP-3 transgene, was used to prime cDNA synthesis from the transgene transcripts. Ten µg total spleen RNA from transgenic founders and controls were incubated with 1 µM of primer at 70°C for 10 min, and placed on ice. The reaction was then supplemented with 10 mM Tris-HCl pH 8.3, 50 mM KCL, 2.5 mM $MgCl_2$, 10 µM of each dNTP, 0.1 mM DTT and 200 U SuperScript II RT. After incubation at 42 °C for 50 min, the reaction was stopped by heating at 72 °C for 15 min. Total RNA were digested by addition of 2 U RNase H and incubation at 37 °C for 20 min. Subsequent PCR reactions were carried out by using murine AGP-3 specific primers. The 5′ PCR primer was

5′ AGC CGC GGC CAC AGG AAC AG 3′

SEQ ID NO: 33

The 3′ PCR primer was

5′ TGG ATG ACA TGA CCC ATA G 3′

SEQ ID NO: 34

The PCR reaction was performed in a volume of 50 µl with 0.5 unit Tag DNA polymerase in 10 mM Tris-HCl pH 8.3, 50 mM KCL, 2.5mM $MgCl_2$, 10 µM of each dNTP, 1 µM of each primer and 1 µl of cDNA product. The reaction was performed at 94 °C for 30 s, 55°C for 30 S, and 72 °C for 1 min, for a total of 35 cycles. The PCR product was analyzed by electrophoresis. Transgene expression was detected in the spleen of all ten AGP-3 transgenic mice founders.

Biological activity of AGP-3

Prior to euthanasia, all animals were weighed, anesthetized by isofluorane and blood was drawn by cardiac puncture. The samples were subjected to hematology and serum chemistry analysis. The serum globulin level in all the AGP-3 transgenic mice (animal 3, 6, 9, 10, 13, 38, 40, 58, 59 and 62) increased more than 100% as compared to the control littermates (animal 7, 8, 11, 12 and 14, Table 1). Total protein level also increased correspondingly in the transgenic group, while albumin level remained the same. No significant differences in other serum chemistry or hematology parameters were observed at this age.

Radiography was performed after terminal exsanguination. There was no difference in the radiodensity or radiologic morphology of the skeleton. Upon gross dissection, major visceral organs were subject to weight analysis. The spleen weight relative to the body weight increased by approximately 45% in the AGP-3 transgenic group as compared to the control mice. The sizes of lymph nodes and Peyer's patches were also increased substantially in all the AGP-3 transgenic mice.

Following gross dissection, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The tissues collected were liver, spleen, pancreas, stomach, the entire gastrointestinal tract, kidney, reproductive organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, esophagus, thyroid, adrenals, urinary

bladder, lymph nodes and skeletal muscle. After fixation, the tissues were processed into paraffin blocks, and 3 μm sections were obtained. All sections were stained with hematoxylin and exosin, and subject to histologic analysis. The size and the number of the follicles in the spleen, lymph nodes and Peyer's patches were increased significantly in the AGP-3 transgenic mice (Figure 5, 6 and 7). The spleen, lymph node and Peyer's patches of both the transgenic and the control mice were subject to immunohistology analysis with B cell and T cell specific antibodies. The formalin fixed paraffin embedded sections were deparaffinized and hydrated to deionized water. The sections were quenched with 3% hydrogen peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat monoclonal anti-mouse B220 and CD3 (Harlan, Indianapolis, IN), respectively. The binding was detected by biotinylated rabbit anti-rat immunoglobulins and peroxidase conjugated streptavidin (BioGenex, San Ramon, CA) with DAB as chromagen (BioTek, Santa Barbara, CA). Sections were counterstained with hematoxylin. The B cell numbers, as indicated by positive B220 staining, increased significantly in the spleen, lymph nodes and Peyer's batches (Figure 5, 6, and 7). The T cell numbers, as indicated by the anti-CD3 staining, were slightly decreased. There were no differences in the morphology of the thymus between the transgenic and the control group. By immunohistology, the T cell population was similar in numbers. At 8 weeks of age, there are no distinctive morphologic changes in the liver, kidneys, or urinary, central nervous, hematopoietic, skeletal, respiratory, gastrointestinal, endocrine, or reproductive systems.

After necropsy, MLN and sections of spleen and thymus from 10 AGP-3 transgenic mice (animals 3, 6, 9, 10, 13, 38, 40, 58, 59 and 62) and 5 control littermates (animals 7, 8, 11, 12, and 14) were removed. Single cell suspensions were prepared by gently grinding the tissues with the flat end

of a syringe against the bottom of a 100 µm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells were washed twice in a 15 ml volume then counted. Approximately 1 million cells from each tissue was stained with 0.5 µg antibody in a 100 µl volume of PBS (without Calcium and Magnesium) + 0.1% Bovine Albumin + 0.01% Sodium Azide. All spleen and MLN samples were incubated with 0.5 µg CD16/32(FcγIII/II) Fc block in a 20 µl volume for 10 minutes prior to the addition of FITC or PE-conjugated monoclonal antibodies against CD90.2 (Thy-1.2), CD45R (B220), CD11b(Mac-1), Gr-1, CD4, or CD8 (PharMingen, San Diego, CA) at 2-8 °C for 30 min. The cells were washed then analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). Thymus samples were stained with FITC conjugated anti-Thy-1.2, FITC conjugated anti-CD4, and PE conjugated anti-CD8 (PharMingen, San Diego, CA).

In the MLN of the AGP-3 transgenic mice, the percentage of B220 positive B cells increased by 100% (Figure 6). The percentage of the Thy-1.2 positive T cells decreased approximately 36%, with similar reductions in both CD4(+) and CD8(+) populations. The helper CD4(+) / suppressor CD8(+) ratio remained unchanged. Similar increases in B cell and reductions in T cell populations were also observed in the spleens of the AGP-3 transgenic mice (Figure 8), though to a lesser extent. No obvious changes in staining with anti-CD11b or anti-Gr-1 antibodies were observed in the lymph node and spleen between the transgenic and the control group. In the thymus, there were no differences in the percentages of Thy-1.2(+), CD4(+), CD8(+) or CD4(+)CD8(+) populations between the AGP-3 transgenic and the control mice.

Serum Immunoglobulin and Autoantibody Analysis

Transgenic mice and control littermates were bled successively at 6, 7, 8, 9, 11, and 12 weeks of age. Serum immunoglobulin levels were

quantitated using by ELISA with Mouse Hybridoma Subtype Kit as suggested by manufacture (Boehringer Mannheim, Indianapolis, IN). Presence of autoantibodies directed against nuclear antigens and dsDNA were examined in the serum by enzyme linked immunosorbant assay (ELISA). The levels of anti-nuclear antibodies were detected using ANA screen kit (Sigma) and anti-mouse IgG peroxidase secondary antibody. Mouse serum samples were diluted 1:200 in ANA screen ELISA. For the detection of anti-dsDNA autoantibodies in serum, high binding ELISA plates were coated with plasmid DNA (Immunovision) as an antigen in the presence of methylated BSA. After blocking the non-specific sites and washing, diluted mouse serum samples were added to wells in duplicated and the binding was quantitated using horse radish peroxidase-labeled anti-mouse IgG or anti-mouse IgM reagents (Southern Biotech). A pooled positive serum from BWF1 mice and pooled negative serum from B6 mice was used as controls. Experiment for the detection of anti-histone antibodies was essentially done similar to anti-DNA ELISA except that carbonate-bicarbonate buffer (pH9.6) buffer was used as coating buffer. Serum antibody data were compared by Mann Whitney test using Sigmastat software (SPSS Science, Chicago, IL).

B Cell Survival and Proliferation Assay

Cells were isolated from spleens of 2-4 months old mice by negative selection. Briefly, B lymphocytes were purified by density gradient centrifugation and then passed over a B cells column (Accurate/ Cedarlane, Westbury, NY). Cells isolated by this method were analyzed by flow cytometry and >90% were found positive for B220 staining. Isolated B cells were cultured in MEM+10% FCS at 37°C, 5%CO_2. Cells were collected from triplicate wells daily on day 1 through day 9 and incubated with 5 μg/ml Propidium Iodide. Cells were analyzed by Flow cytometry and the percentage of dead cells was calculated. For B cell

proliferation assay, purified (10^5) B cells from B6 mice as described above were cultured in MEM+10% heat inactivated FCS in triplicate in 96 well flat bottomed plate with/without 2 µg/ml of Goat F(ab')$_2$ anti-mouse IgM (Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania) and/or indicated amount of recombinant AGP-3 for a period of 4 days at 37 °C, 5%CO_2. Proliferation was measured by an uptake of radioactive ^{3}(H) thymidine in last 18 hours of pulse. Data is shown in figure 14 as mean±standard deviation of triplicate wells.

-3; 260-3.

B Cell Hyperplasia and Hypergammaglobulinemia in AGP-3 Transgenic Mice

To gain insights into the biological function for AGP-3, transgenic mice were generated that expressed full-length murine AGP-3 protein driven by the ubiquitous 3-actin promoter. Founder mice harboring the AGP-3 transgene were identified by PCR analysis of genomic DNA samples. Transgene expression was confirmed by RT-PCR from spleen total RNA. At 8 weeks of age, ten AGP-3 transgenic mice and five control littermates were subject to necropsy and pathological analysis. The transgenic mice were of normal size and weight. However, the spleen weight relative to the body weight increased by approximately 45% in the AGP-3 transgenic group as compared to the control mice. The sizes of lymph nodes and Payer's Patches were also increased substantially in all the AGP-3 transgenic mice. Histology analysis demonstrated that the size and the number of the follicles in the spleen, lymph nodes and Payer's patches were increased significantly in the AGP-3 transgenic group (Figure 10). Immunohistology staining with B and T cell specific markers indicated the B cell numbers increased significantly in the spleen, lymph nodes and Payer's patches of the transgenic group (Figure 10). The T cell numbers, as indicated by the anti-CD3 staining, were decreased

correspondingly (Figure 10). There were no differences in the morphology and immunostaining of thymus between the transgenic and the control groups. No changes were observed in other organs or organ systems of the 8 weeks old transgenic mice, including kidney, liver, and hematopoietic tissues.

The B cell hyperplasia phenotype in the AGP-3 transgenic mice was also confirmed by flow cytometry analysis. In the mesenteric lymph nodes of the AGP-3 transgenic mice, the percentage of B220 positive B cells increased by 100% (Figure 11). The percentage of the Thy-1.2 positive T cells decreased by approximately 36%, with similar reductions in both CD4(+) and CD8(+) T cells. Similar increase in B cell and reduction in T cell populations were also observed in the spleens of the AGP-3 transgenic mice, though to a lesser extent (Figure 11). Of note, the total T cell numbers in the lymph node and spleen of AGP-3 transgenic mice were similar to the control littermates. In the thymus, there were no differences in the percentages of single positive CD4(+) or CD8(+) T cells, or CD4(+)CD8(+) populations between the AGP-3 transgenic and the control mice (Figure 11). No obvious changes in staining with anti-CD11b or anti-Gr-1 antibodies were observed in the lymph nodes and spleen between the transgenic and the control group (Figure 11). The histological and FACS analysis, together, suggested severe B cell hyperplasia phenotype in the AGP-3 transgenic mice.

We also examined B cell populations of different developmental stages by FACS analysis. No differences were observed in the percentage of the pro B (B220+IgM-), immature B (B220+IgM+), or mature B (IgM+IgD+) within spleenic B cell population of the AGP-3 transgenic mice as compared to the control littermates. In addition, the number of the spleenic CD5+ B cells in the AGP-3 transgenic mice from 1 to 9 month of age was unaltered. We also didn't detect any alteration of the CD40

expression level on B cells in the transgenic mice, suggesting that the B cell hyperplasia in the AGP-3 transgenic mice was not caused by CD40 upregulation.

In addition to the B cell hyperplasia phenotype, the AGP-3 transgenic mice also had severe hypergammaglobulinemia . The serum globulin level in AGP-3 transgenic mice increased more that 100% as compared to the control group. Total protein level also increased correspondingly in the transgenic, while albumin level remained the same. The increased B cell numbers and high serum globulin level suggested elevated serum immunoglobulin titer. Thus we examined serum levels of IgM, IgG, IgA and IgE of AGP-3 transgenic mice from 6 to 12 weeks of age. Comparing to the same age control littermates, serum IgM, IgG, IgA and IgE were significantly increased in all age groups of AGP-3 transgenic mice. The increase found in serum IgG was not specific to any particular subclass (IgG1, IgG2a, IgG2b, and IgG3). No significant differences in other serum chemistry or hematology parameters were observed at this age. The increased serum immunoglobulin levels is likely to result directly from increased B cell number, but may also be aggravated by increased B cell antibody production.

Autoantibodies associated with lupus in AGP-3 transgenic mice

Increased humoral immunity in AGP-3 transgenic mice warranted us to look for possible phenotypes resembling B cell associated autoimmune diseases such as systemic lupus erythematosus (SLE). The common denominator in lupus patients and lupus prone mice is IgG autoantibody production, and the hallmark of this disease is the presence of elevated anti-nuclear antibodies in the serum. The emergence of anti-DNA antibodies represents one final outcome in the different murine lupus models and patients with SLE. When sera from transgenic and non-transgenic mice at various age were examined for the presence of

autoantibodies recognizing nuclear antigens or dsDNA, two different lines of AGP-3 transgenic mice began to show presence of autoantibodies at around 8 weeks of age (Table 1). The amount of anti-nuclear and anti-dsDNA antibody increased with their age in the transgenic animals (Table 1). More interestingly, at 5 and 8 months of age, AGP-3 transgenic mice showed 5-10 higher amount of anti-dsDNA antibodies compared to age matched lupus prone (NZBxNZW)F1 mice. The presence of autoantibodies in the serum of AGP-3 transgenic mice did not discriminate between gender of mice. Both IgG and IgM antibodies to dsDNA were detected in transgenic animals. Presence of such autoantibodies was undetectable in non-transgenic littermates, as expected.

Immune Complex Deposits in the Kidney of AGP-3 Transgenic Mice

Presence of anti-DNA antibodies followed by immune complex induced renal damage is classical picture seen in lupus associated nephritis. At 5 month of age, the AGP-3 transgenic mice developed glomerular proteinaceous deposits in the kidney (Figure 13). The deposits were seen in more than 60% of the glomeruli in the transgenic mice, but absent in the control littermates. Immunohistology showed the deposits contained moderate amounts of IgG and larger amounts of IgM (Figure 13). Trichrome staining showed no deposit of connective tissues in the glomeruli at 5 month of age. There is also no evidence of any cellular proliferation or presence of inflammatory cells at this age (Figure 13). Interestingly, the kidney lesions progressed as the transgenic mice grew older. At 8 month of age, there was obvious enlargement of glomeruli in the AGP-3 transgenic mice as compared to the age matched control littermates (Figure 13G). In addition, we also detected extensive connective tissue deposits in the enlarged glomeruli (Figure 13G).

Comparing to the 5 month old mice, the 8 month old transgenic mice had increased IgG level in the glomeruli immune complex deposits (Figure 13I). Majority of the glomeruli in the AGP-3 transgenic mice were affected. We also performed serum and urine chemistry analysis of 5 month old and 8 month old AGP-3 transgenic along with the control littermates. No significant differences were noticed in the 5 month old AGP-3 transgenic mice. However, in the 8 month old mice, we observed increases in serum blood urea nitrogen (BUN) and calcium levels and decrease in serum phosphate level. In addition, the 8 month old AGP-3 mice also had increased protein level in the urine. These changes, together, suggest the onset of renal failure in the 8 month old AGP-3 transgenic mice. In conclusion, the high serum autoantibodies followed by the kidney lesions in theAGP-3 transgenic mice clearly resemble to the pathological progression in the SLE patients and lupus prone mice.

AGP-3 Stimulates B Cell Survival and Proliferation : a Possible Mechanism for Autoimmunity

The B cell hyperplasia phenotype in the AGP-3 transgenic mice might arise from increased B cell survival and/or increased B cell proliferation. We first compared the viability of B cells from AGP-3 transgenic mice with that of the control littermates. B cells were isolated from both transgenic or control mice and incubated in minimal essential medium supplemented with 10% heat inactivated fetal bovine serum. Viability of the B cells was measured by FACS analysis for Propidium Iodide uptake (Figure 14A). By day 3, 30% of B cells isolated from the control mice were dead, whereas only 10% of B cells from AGP-3 transgenic mice were dead. By day 5, 70% of B cells from AGP-3 mice were still viable, whereas only 15% of B cell from control littermates were viable. By day 9, almost 50% of the AGP-3 transgenic B cells still remained viable. Therefore, transgenic expression of AGP-3 prolonged B cell

viability. It remains to be determined if this B cell survival stimuli result directly from AGP-3 action on B cells or through its modulation of the immune system.

Recently Schneider et al (Schneider et al., 1999, and Moore et al., 1999) reported co-stimulation of B cell proliferation by BAFF/BLYS with anti-IgM. We found that AGP-3 alone can also stimulates B cell proliferation in a dose dependent manner with an ED_{50} of approximately 3ng/ml (Figure 14B, upper). A ten fold increase of B cell proliferation was detected by AGP-3 treatment at 10 ng/ml concentration as compared to the untreated cells. In our experiment, anti-IgM alone at 2 μg/ml concentration increased B cell proliferation by 24 fold. Treatment with anti-IgM (2 μg/ml) in combination with various doses of AGP-3 led to dose dependent increase of B cell proliferation, with a maximal 13 fold increase as compared anti-IgM treatment alone and a total of 320 fold increase as compared to the untreated cells. Thus, AGP-3 is a potent B cell stimulatory factor. The increased B cell survival and proliferation may together contribute to the B cell hyperplasia and autoimmune lupus like changes in the AGP-3 transgenic mice.

Table 1: Lupus associated autoantibodies in the serum of AGP-3 transgenic mice.

Autoantibodies	Age (months)	AGP-3 tg (n)	Non-tg littermates (n)	p value
Antinuclear antibodies (IgG)[a]	2-3	7^ (9)	1*(8)	
	5-6	9 (9)	1*(8)	
	8-9	8 (8)	1*(6)	
Anti-dsDNA (IgG)[b]	<2	697±284 (7)	277±67 (7)	NS
	3-4	842±351 (7)	235±49 (7)	<.005
	6-7	2515±428 (5)	970±344 (7)	<.019
	8-10	12293±6767 (11)	1070±602 (12)	<.017
Anti-dsDNA (IgM)[b]	<2	275±33 (7)	46±5 (7)	<.001
	3-4	1684±920 (7)	63±13 (7)	<.003
	6-7	6998±5515 (5)	98±14 (7)	<.001
	8-10	13712±9147 (11)	79±14 (12)	<.001
Anti-Histone (Ig)[b]	<2	741±264 (7)	52±8 (7)	<.001
	3-4	837±436 (7)	53±14 (7)	<.003
	6-7	4220±933 (5)	60±10 (7)	<.001
	8-10	16555±4618 (11)	295±173 (12)	<.001

^ includes two weak positive.

* Weak positive

a: Data is shown as number of ANA positive (mean+2sd of transgene negative littermates) mice using ANA screen kit.

b: Data is represented as mean±SE for each group. Values are shown as Units/ml.

NS: not significant

Bacterial Expression of AGP-3 protein

PCR amplification employing the primer pairs and templates described below are used to generate various forms of human AGP3 proteins. One primer of each pair introduces a TAA stop codon and a unique XhoI or SacII site following the carboxy terminus of the gene. The other primer of each pair introduces a unique NdeI site, a N-terminal methionine, and optimized codons for the amino terminal portion of the gene. PCR and thermocycling is performed using standard recombinant DNA methodology. The PCR products are purified, restriction digested, and inserted into the unique NdeI and XhoI or SacII sites of vector pAMG21 (ATCC accession no. 98113) and transformed into the prototrophic E. coli 393 or 2596. Other commonly used E. coli expression vectors and host cells are also suitable for expression. After transformation, the clones are selected, plasmid DNA is isolated and the sequence of the AGP3 binding protein insert is confirmed.

pAMG21-Human AGP3 protein [128-285]

This construct was engineered to be C-terminal 158 amino acids of human AGP3 and have the following N-terminal and C-terminal residues:

NH_2-MNSRNKR ——GALKLL-COOH.

SEQ ID NO: 35

The template to be used for PCR was human AGP3 in pCDNA3.1(+) vector. Oligonucleotides #1761-31 and #1761-33 were the primer pair to be used for PCR and cloning this gene construct.

1761-31:

5'-ATT TGA TTC TAG AAG GAG GAA TAA CAT ATG AAC AGC CGT AAT AAG CGT GCC GTT CAG GGT -3'

(SEQ ID NO:36)

1761-33:

5'-CCG CGG ATC CTC GAG TTA CAG CAG TTT CAA TGC ACC AAA AAA TGT -3'

(SEQ ID NO:37)

pAMG21-Human FLAG-AGP3 protein [128-285]

This construct was engineered to be C-terminal 158 amino acids of human AGP3 preceded with FLAG epitope. The construct encoded following following N-terminal and C-terminal residues:

NH_2-MDYKDDDDKKLNSRNKR------GALKLL-COOH

(SEQ ID NO: 38)

The template to be used for PCR was human AGP3 in pCDNA3.1(+) vector. Oligonucleotides #1761-32 and #1761-33 were the primer pair to be used for PCR and cloning this gene construct.

1761-32:

5'-GAC GAT GAC AAG AAG CTT AAC AGC CGT AAT AAG CGT GCC GTT CAG GGT -3'

(SEQ ID NO:39)

1761-33:

5'-CCG CGG ATC CTC GAG TTA CAG CAG TTT CAA TGC ACC AAA AAA TGT -3'

(SEQ ID NO:37)

E. coli were induced during fermentation, the lysate was applied to Q Sepharose FF (Pharmacia, Piscaataway, NJ) equilibrated in 10 mM Mes pH 6.0 and eluted with 50- 400 mM NaCl gradient over 30 column volumes. Fractions containing AGP-3 were pooled and loaded onto a Q Sepharose HP column (Pharmacia, Piscataway, NJ) equilibrated in 10 mM Tris-HCL pH 8.5. AGP-3 was eluted with an increasing linear NaCl gradient (50 mM-200 mM) over 30 column volumes. Endotoxin was removed by application to Sp HiTRAP column (Pharmacia, Piscataway, NJ) pH 4.8 and eluted with 100-500 mM NaCl in 10 mM sodium acetate pH 4.8 over 25 column volumes. Final endotoxin level of the purified

protein is approximately 0.2 EU/mg. The purified human AGP-3 is truncated at residue Arg133 as indicated by N-terminal sequencing and has a molecular weight of 16.5 KDa by reducing SDS-PAGE. The purified human FLAG-AGP-3 protein is confirmed by N-terminal sequence analysis of the protein. The FLAG-AGP3 protein is recognized by M2 monoclonal antibody against FLAG epitope (Kodak, New Haven, CT).

For europium labeling of the protein, human AGP-3 (lot# 092299) was dialyzed into 50 mM sodium carbonate pH 9. Europium labeling reagent (Wallac Delfia reagent lot# 704394) was dissolved in the same buffer. AGP-3 protein was mixed with a 20-fold molar excess of labeling reagent for 24 hours at room temperature. The mixture was then placed on a Sephadex G-25 column which had been equilibrated in 50 mM Tris-HCl pH 7.8, 150 mM NaCl. The protein was eluted from the column with the same buffer. Protein concentration was determined using the BCA method (Pierce Chemical Co.).

Abbreviations

Abbreviations as used throughout this specification are defined as follows, unless otherwise defined in specific instances.

CDR	complementarity determining region
dsDNA	double-stranded DNA
EST	expressed sequence tag
ORF	open reading frame
SDS	sodium dodecyl sulfate
TNF	tumor necrosis factor

* * *

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the

appended claims cover all such equivalent variations that come within the scope of the invention as claimed.

What is claimed is:

1. An isolated or recombinant polypeptide having a sequence comprising SEQ ID NO: 25, wherein said polypeptide does not comprise SEQ ID NOS: 2, 4, or 5 or a sequence with 90% identity thereto.
2. The polypeptide of Claim 1, further comprising SEQ ID NO: 26.
3. The polypeptide of Claim 1 having a sequence comprising SEQ ID NO: 27.
4. The polypeptide of Claim 1, wherein said polypeptide does not comprise SEQ ID NOS: 2, 4, or 5 or a sequence with 80% identity to SEQ ID NOS: 2, 4, or 5.
5. The polypeptide of Claim 1, comprising an Fc-region.
6. The polypeptide of Claim 1, wherein the polypeptide has the structure

$$(X^1)_a\text{-}F^1\text{-}(X^2)_b$$

wherein:

F^1 is a vehicle;

X^1 and X^2 are each independently selected from $-(L^1)_c\text{-}P^1$, $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2$, $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3$, and $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3\text{-}(L^4)_f\text{-}P^4$

P^1, P^2, P^3, and P^4 are each independently selected from SEQ ID NOS: 6, 25, 26, and 27;

L^1, L^2, L^3, and L^4 are each independently linkers; and

a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1.

7. The composition of matter of Claim 6 of the formula

$$X^1\text{-}F^1$$

or

$$F^1\text{-}X^2.$$

8. The composition of matter of Claim 6 of the formula

$$F^1\text{-}(L^1)_c\text{-}P^1.$$

9. The composition of matter of Claim 6 of the formula

$$F^1\text{-}(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2.$$

10. The composition of matter of Claim 6 wherein F^1 is an IgG Fc domain.

11. The composition of matter of Claim 6 wherein F^1 is an IgG1 Fc domain.

12. The polypeptide of Claim 1, wherein the polypeptide comprises an antibody sequence in which one or more amino acids from antibody CDR regions are replaced by sequences selected from SEQ ID NOS: 6, 25, 26, and 27.

13. The polypeptide of Claim 12, wherein a first CDR region is replaced by SEQ ID NO: 25 and a second CDR region is replaced by SEQ ID NO: 26.

14. The polypeptide of Claim 1, wherein the polypeptide comprises a sequence selected from SEQ ID NOS: 7 TO 24 and 40 shown in Figure 9, except that the B/B' region is replaced by SEQ ID NO: 25.

15. The polypeptide of Claim 1, wherein the polypeptide comprises a sequence selected from SEQ ID NOS: 7 TO 24 and 40 shown in Figure 9, except that the B/B' region is replaced by SEQ ID NO: 25 and the E/F region is replaced by SEQ ID NO: 26.

16. The polypeptide of Claim 1, wherein the polypeptide comprises a sequence selected from SEQ ID NOS: 7 TO 24 and 40 shown in Figure 9, except that the B/I region is replaced by SEQ ID NO: 27.

17. A polypeptide of Claim 1 capable of eliciting B cell growth, survival, or activation in mesenteric lymph nodes.

18. The protein of any of Claims 1, 2, 3, 4, 14, 15, 16, or 17, wherein the protein is covalently linked to a water-soluble polymer or a carbohydrate.

19. The protein of Claim 18, wherein the polymer is polyethylene glycol.

20. The protein of Claim 18, wherein the carbohydrate is dextran.

21. An isolated nucleic acid encoding a protein of any of Claims 1 to 17.

22. The nucleic acid of Claim 21 including one or more codons preferred for Escherichia coli expression.

23. The nucleic acid of Claim 21 having a detectable label attached thereto.

24. An expression vector comprising the nucleic acid of Claim 21.

25. A host cell transformed or transfected with the expression vector of Claim 24.

26. The host cell of Claim 25, wherein the cell is a prokaryotic cell.

27. The host cell of Claim 26, wherein the cell is Escherichia coli.

28. A method to assess the ability of a candidate compound to bind to an AGP-3 related protein comprising:

(a) incubating a polypeptide of Claim 1 with the candidate compound under conditions that allow binding; and

(b) measuring the bound compound.

29. A method of regulating expression of an AGP-3 related protein in an animal comprising administering to the animal a nucleic acid complementary to the nucleic acid of Claim 22.

30. A pharmaceutical composition comprising a therapeutically effective amount of a protein of Claim 1 in a pharmaceutically acceptable carrier, adjuvant, solubilizer, stabilizer and/or anti-oxidant.

31. A method of modulating B cell growth, survival, or activation in a mammal, which comprises administering a therapeutically effective amount of a modulator of an AGP-3 related protein.

32. The method of Claim 31, wherein the B cell growth, survival, or activation takes place in mesenteric lymph nodes.

33. An antibody that specifically binds to SEQ ID NOS: 25, 26, or 27.

34. The antibody of claim 33, wherein the antibody is a monoclonal antibody.

35. The antibody of claim 33, wherein the antibody was generated by phage display.

36. A method of modulating B cell growth, survival, or activation in a mammal comprising administering a therapeutically effective amount of the antibody of Claim 33.

37. The method of Claim 36, wherein the B cell growth, survival, or activation takes place in mesenteric lymph nodes.

38. A method to modulate hybridoma cell antibody production, which comprises treating hybridoma cells with the compound of Claim 1.

FIGURE 1

Human AGP3

```
          10                  30                  50
GAATTCGGCACGAGCTGAGGGGTGAGCCAAGCCCTGCCATGTAGTGCACGCAGGACATCA
          70                  90                 110
ACAAACACAGATAACAGGAAATGATCCATTCCCTGTGGTCACTTATTCTAAAGGCCCCAA
         130                 150                 170
CCTTCAAAGTTCAAGTAGTGATATGGATGACTCCACAGAAAGGGAGCAGTCACGCCTTAC
                        M   D   D   S   T   E   R   E   Q   S   R   L   T
         190                 210                 230
TTCTTGCCTTAAGAAAAAGAGAAGAAATGAAACTGAAGGAGTGTGTTTCCATCCTCCCACG
  S   C   L   K   K   R   E   E   M   K   L   K   E   C   V   S   I   L   P   R
         250                 270                 290
GAAGGAAAGCCCCTCTGTCCGATCCTCCAAAGACGGAAAGCTGCTGGCTGCAACCTTGCT
  K   E   S   P   S   V   R   S   S   K   D   G   K   L   L   A   A   T   L   L
         310                 330                 350
GCTGGCACTGCTGTCTTGCTGCCTCACGGTGGTGTCTTTCTACCAGGTGGCCGCCCTGCA
  L   A   L   L   S   C   C   L   T   V   V   S   F   Y   Q   V   A   A   L   Q
         370                 390                 410
AGGGGACCTGGCCAGCCTCCGGGCAGAGCTGCAGGGCCACCACGCGGAGAAGCTGCCAGC
  G   D   L   A   S   L   R   A   E   L   Q   G   H   H   A   E   K   L   P   A
         430                 450                 470
AGGAGCAGGAGCCCCCAAGGCCGGCCTGGAGGAAGCTCCAGCTGTCACCGCGGGACTGAA
  G   A   G   A   P   K   A   G   L   E   E   A   P   A   V   T   A   G   L   K
         490                 510                 530
AATCTTTGAACCACCAGCTCCAGGAGAAGGCAACTCCAGTCAGAACAGCAGAAATAAGCG
  I   F   E   P   P   A   P   G   E   G   N   S   S   Q   N   S   R   N   K   R
         550                 570                 590
TGCCGTTCAGGGTCCAGAAGAAACAGTCACTCAAGACTGCTTGCAACTGATTGCAGACAG
  A   V   Q   G   P   E   E   T   V   T   Q   D   C   L   Q   L   I   A   D   S
         610                 630                 650
TGAAACACCAACTATACAAAAAGGATCTTACACATTTGTTCCATGGCTTCTCAGCTTTAA
  E   T   P   T   I   Q   K   G   S   Y   T   F   V   P   W   L   L   S   F   K
         670                 690                 710
AAGGGGAAGTGCCCTAGAAGAAAAAGAGAATAAAATATTGGTCAAAGAAACTGGTTACTT
  R   G   S   A   L   E   E   K   E   N   K   I   L   V   K   E   T   G   Y   F
         730                 750                 770
TTTTATATATGGTCAGGTTTTATATACTGATAAGACCTACGCCATGGGACATCTAATTCA
  F   I   Y   G   Q   V   L   Y   T   D   K   T   Y   A   M   G   H   L   I   Q
         790                 810                 830
GAGGAAGAAGGTCCATGTCTTTGGGGATGAATTGAGTCTGGTGACTTTGTTTCGATGTAT
  R   K   K   V   H   V   F   G   D   E   L   S   L   V   T   L   F   R   C   I
         850                 870                 890
TCAAAATATGCCTGAAACACTACCCAATAATTCCTGCTATTCAGCTGGCATTGCAAAACT
  Q   N   M   P   E   T   L   P   N   N   S   C   Y   S   A   G   I   A   K   L
         910                 930                 950
GGAAGAAGGAGATGAACTCCAACTTGCAATACCAAGAGAAAATGCACAAATATCACTGGA
  E   E   G   D   E   L   Q   L   A   I   P   R   E   N   A   Q   I   S   L   D
         970                 990                1010
TGGAGATGTCACATTTTTTGGTGCATTGAAACTGCTGTGACCTACTTACACCATGTCTGT
  G   D   V   T   F   F   G   A   L   K   L   L
        1030                1050                1070
AGCTATTTTCCTCCCTTTCTCTGTACCTCTAAGAAGAAAGAATCTAACTGAAAATACCAA
        1090                1110                1130
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTAGTTAAAAAAAAAAAAAAAAAAAAAAAAA
        1150                1170
AAAAAAAAAAAAAAAAAAAAAACTCGGAGGGGG
```

FIGURE 2

Mouse AGP3

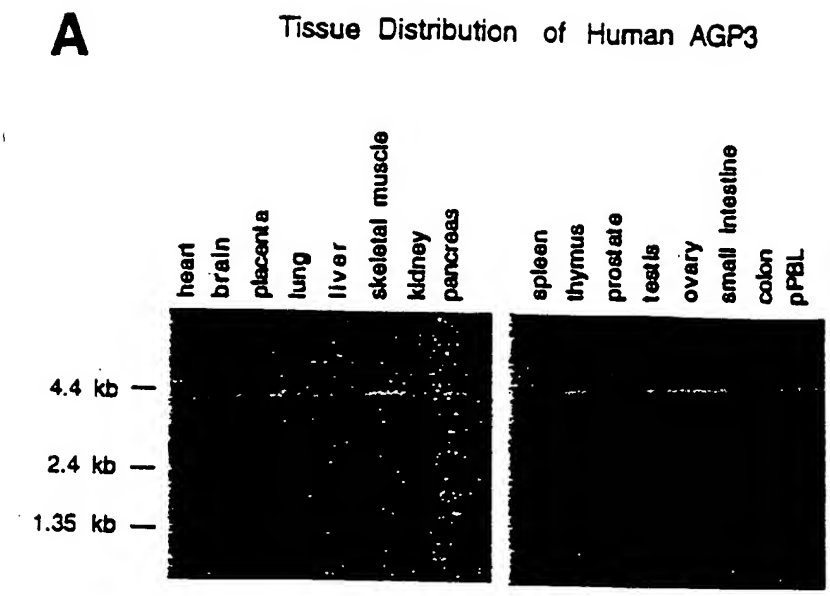
```
          10                  30                  50
GAATTCGGCACGAGCTCCAAAGGCCTAGACCTTCAAAGTGCTCCTCGTGGAATGGATGAG
                                                  M  D  E
          70                  90                 110
TCTGCAAAGACCCTGCCACCACCGTGCCTCTGTTTTTGCTCCGAGAAAGGAGAAGATATG
S  A  K  T  L  P  P  P  C  L  C  F  C  S  E  K  G  E  D  M
         130                 150                 170
AAAGTGGGATATGATCCCATCACTCCGCAGAAGGAGGAGGGTGCCTGGTTTGGGATCTGC
K  V  G  Y  D  P  I  T  P  Q  K  E  E  G  A  W  F  G  I  C
         190                 210                 230
AGGGATGGAAGGCTGCTGGCTGCTACCCTCCTGCTGGCCCTGTTGTCCAGCAGTTTCACA
R  D  G  R  L  L  A  A  T  L  L  L  A  L  L  S  S  S  F  T
         250                 270                 290
GCGATGTCCTTGTACCAGTTGGCTGCCTTGCAAGCAGACCTGATGAACCTGCGCATGGAG
A  M  S  L  Y  Q  L  A  A  L  Q  A  D  L  M  N  L  R  M  E
         310                 330                 350
CTGCAGAGCTACCGAGGTTCAGCAACACCAGCCGCCGCGGGTGCTCCAGAGTTGACCGCT
L  Q  S  Y  R  G  S  A  T  P  A  A  A  G  A  P  E  L  T  A
         370                 390                 410
GGAGTCAAACTCCTGACACCGGCAGCTCCTCGACCCCACAACTCCAGCCGCGGCCACAGG
G  V  X  L  L  T  P  A  A  P  R  P  H  N  S  S  R  G  H  R
         430                 450                 470
AACAGACGCGCTTTCCAGGGACCAGAGGAAACAGAACAAGATGTAGACCTCTCAGCTCCT
N  R  R  A  F  Q  G  P  E  E  T  E  Q  D  V  D  L  S  A  P
         490                 510                 530
CCTGCACCATGCCTGCCTGGATGCCGCCATTCTCAACATGATGATAATGGAATGAACCTC
P  A  P  C  L  P  G  C  R  H  S  Q  H  D  D  N  G  M  N  L
         550                 570                 590
AGAAACATCATTCAAGACTGTCTGCAGCTGATTGCAGACAGCGACACGCCGACTATACGA
R  N  I  I  Q  D  C  L  Q  L  I  A  D  S  D  T  P  T  I  R
         610                 630                 650
AAAGGAACTTACACATTTGTTCCATGGCTTCTCAGCTTTAAAAGAGGAAATGCCTTGGAG
K  G  T  Y  T  F  V  P  W  L  L  S  F  K  R  G  N  A  L  E
         670                 690                 710
GAGAAAGAGAACAAAATAGTGGTGAGGCAAACAGGCTATTTCTTCATCTACAGCCAGGTT
E  K  E  N  K  I  V  V  R  Q  T  G  Y  F  F  I  Y  S  Q  V
         730                 750                 770
CTATACACGGACCCCATCTTTGCTATGGGTCATGTCATCCAGAGGAAGAAAGTACACGTC
L  Y  T  D  P  I  F  A  M  G  H  V  I  Q  R  K  k  V  H  V
         790                 810                 830
TTTGGGGACGAGCTGAGCCTGGTGACCCTGTTCCGATGTATTCAGAATATGCCCAAAACA
F  G  D  E  L  S  L  V  T  L  F  R  C  I  Q  N  M  P  K  T
         850                 870                 890
CTGCCCAACAATTCCTGCTACTTGGCTGGCATCGCGAGGCTGGAAGAAGGAGATGGATT
L  P  N  N  S  C  Y  S  A  G  I  A  R  L  E  E  G  D  E  I
         910                 930                 950
CAGCTTGCAATTCCTCGGGAGAATGCACAGATTCACGCAACGGAGACGACACCTTCTTT
Q  L  A  I  P  R  E  N  A  Q  I  S  R  N  G  D  D  T  F  F
         970                 990                1010
GGTGCCCTAAAACTGCTGTAACTCACTTGCTGGAGTGCGTGATCCCCTTCCCTCGTCTTC
G  A  L  K  L  L
        1030                1050                1070
TCTGTACCTCCGAGGGAGAAACAGACGACTGGAAAAATAAAAGATGGGGAAAGCCGTCA
        1090                1110                1130
GCGAAAGTTTTCTCGTGACCCGTTGAATCTGATCCAAACCAGGAAATATAACAGACAGCC
        1150                1170                1190
```

FIGURE 3

Alignment of human and murine AGP3 protein sequences

```
Hagp3   MDDSTER.EQ SRLTSCLKKR EEMKLKECVS ILPRKESPSV RSSKDGKLLA
Magp3   MDESAKTLPP PCLCFCSEKG EDMKVGYDPI TPQKEEGAWF GICRDGRLLA
cons    MD.S ..... L  C  K. E. MK ..... E . ..... DG. LLA

Hagp3   ATLLLALLSC CLTVVSFYQV AALQGDLASL RAELQGHHAE KLPAGAGAPK
Magp3   ATLLLALLSS SFTAMSLYQL AALQADLMNL RMELQSYRGS ATPAAAGAPE
cons    ATLLLALLS. .. T    S. YQ. AALQ. DL   L R. ELQ          PA. AGAP.

Hagp3   AGLEEAPAVT AGLKIFEPPA PGEGNSSQNS RNKRAVQGPE ET .....
Magp3              LT AGVKLLTPAA PRPHNSSRGH RNRRAFQGPE ETEQDVDLSA
cons    ..... T AG.  K    P. A P    NSS      RN. RA. QGPE ET

                                                    B
Hagp3   .....                                VTQDCLQ LIADSETPTI QKGSYTFVPW
Magp3   PPAPCLPGCR HSQHDDNGMN LRNIIQDCLQ LIADSDTPTI RKGTYTFVPW
cons    ..... .....  .....QDCLQ LIAD.TPTI .KG.YTFVPW

        B'        C'        C'          D'                E
Hagp3   LLSFKRGSAL EEKENKILVK ETGYFFIYGQ VLYTDKTYAM GHLIQRKKVH
Magp3   LLSFKRGSAL EEKENKIVVR QTGYFFIYSQ VLYTDPIFAM GHVIQRKKVH
cons    LLSFKRG.AL EEKENKI.V.  .TGYFFIY.Q VLYTD.....AM GH.IQRKKVH

                F                                G              H
Hagp3   VFGDELSLVT LFRCIQNMPE TLPNNSCYSA GIAKLEEGDE LQLAIPRENA
Magp3   VFGDELSLVT LFRCIQNMPK TLPNNSCYSA GIAKLEEGDE LQLAIPRENA
__cons  VFGDELSLVT LFRCIQNMP. TLPNNSCYSA GIA.LEEGDE .QLAIPRENA

                    I
Hagp3   . QISLDGDVTF FGALKLL
Magp3   QISRNGDDTF FGALKLL
cons    QIS.. GD.TF FGALKLL
```

FIGURE 4


A
Tissue Distribution of Human AGP3
heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
spleen
thymus
prostate
testis
ovary
small intestine
colon
pPBL
4.4 kb
2.4 kb
1.35 kb

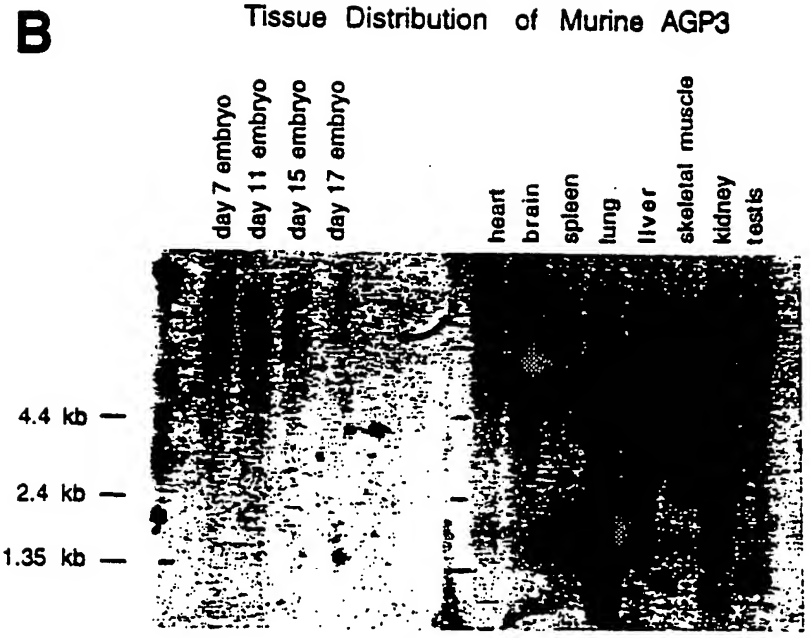
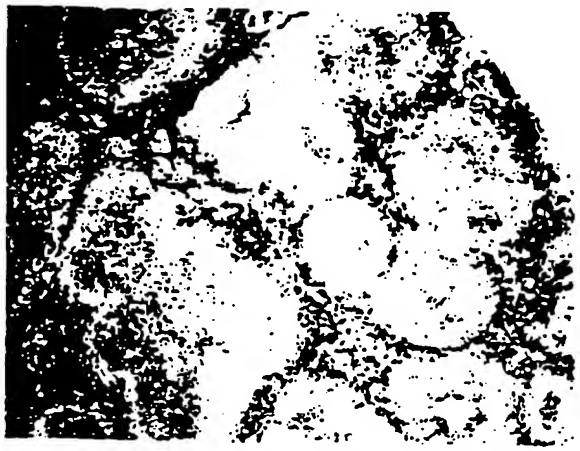

B
Tissue Distribution of Murine AGP3
day 7 embryo
day 11 embryo
day 15 embryo
day 17 embryo
heart
brain
spleen
lung
liver
skeletal muscle
kidney
testis
4.4 kb
2.4 kb
1.35 kb

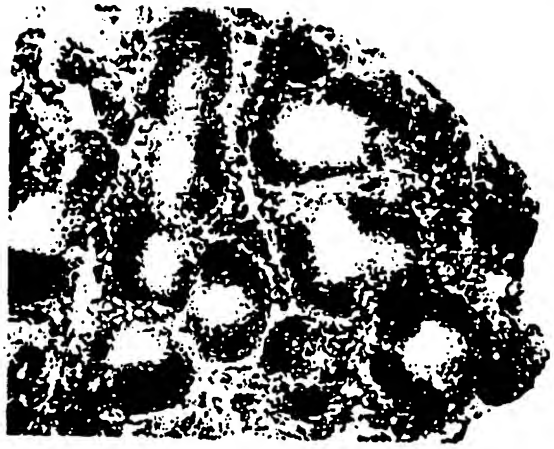
FIGURE 5

A

98M297 HH5 - control spleen, H&E, 2x

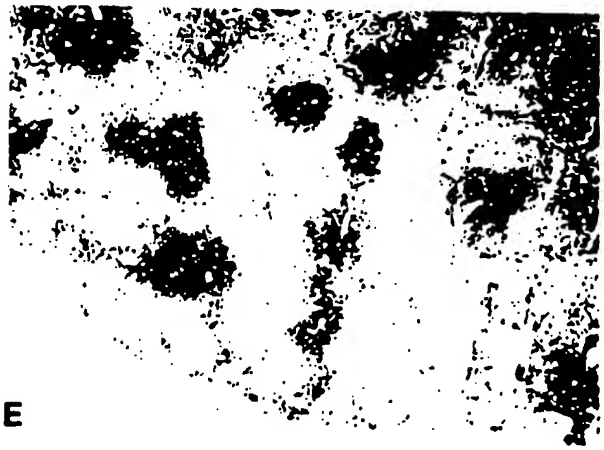


98M297 HH5 - expressor spleen, H&E, 2x



98M297 HH5 - control spleen, B220, 2x

98M297 HH5 - expressor spleen, B220, 2x

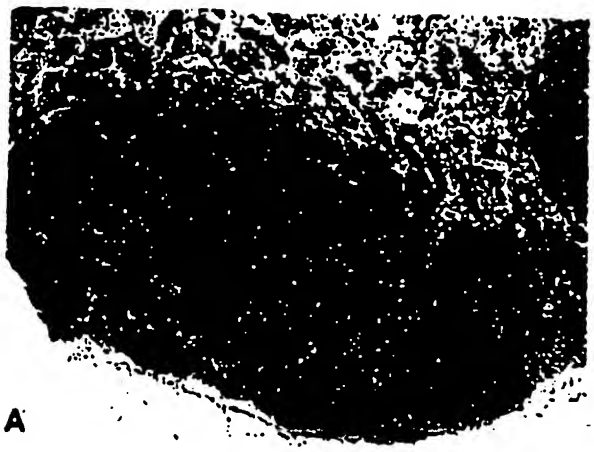


E

98M297 HH5 - control spleen, CD3, 2x

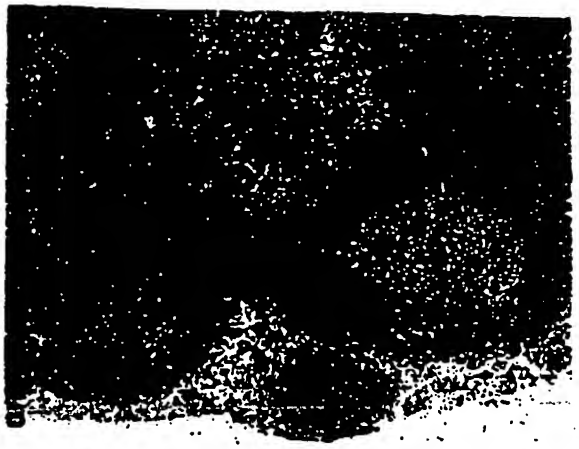
98M297 HH5 - expressor spleen, CD3, 2x

FIGURE 6



A

98M297 HH5 - control lymph node H&E, 4x



98M297 HH5 - expressor lymph node H&E, 4x

C

98M297 HH5 - control lymph node B220, 4x

D

98M297 HH5 - expressor lymph node B220, 4x

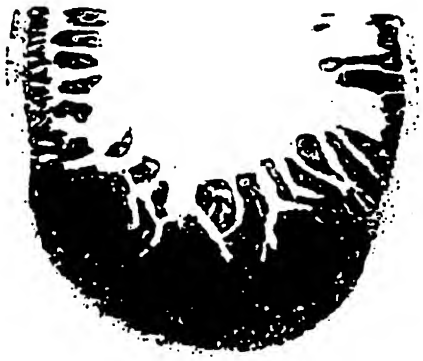
E

98M297 HH5 - control lymph node CD3, 4x

F

98M297 HH5 - expressor lymph node CD3, 4x

FIGURE 7



A

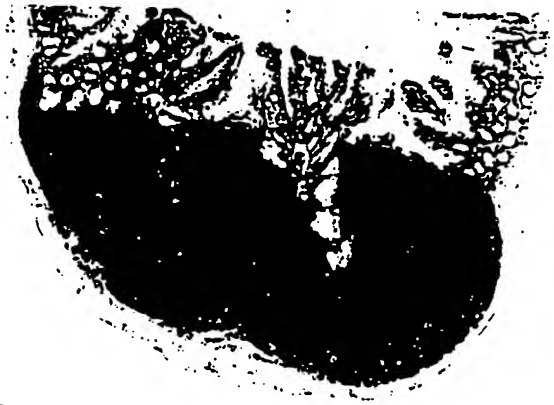
98M297 HH5 - control Payer's patch H&E, 4x

B

98M297 HH5 - expressor Payer's patch H&E, 4x

C

98M297 HH5 - control Payer's patch B220, 4x



D

98M297 HH5 - expressor Payer's patch B220, 4x

E

98M297 HH5 - control Payer's patch CD3, 4x

F

98M297 HH5 - expressor Payer's patch CD3, 4x

FIGURE 8

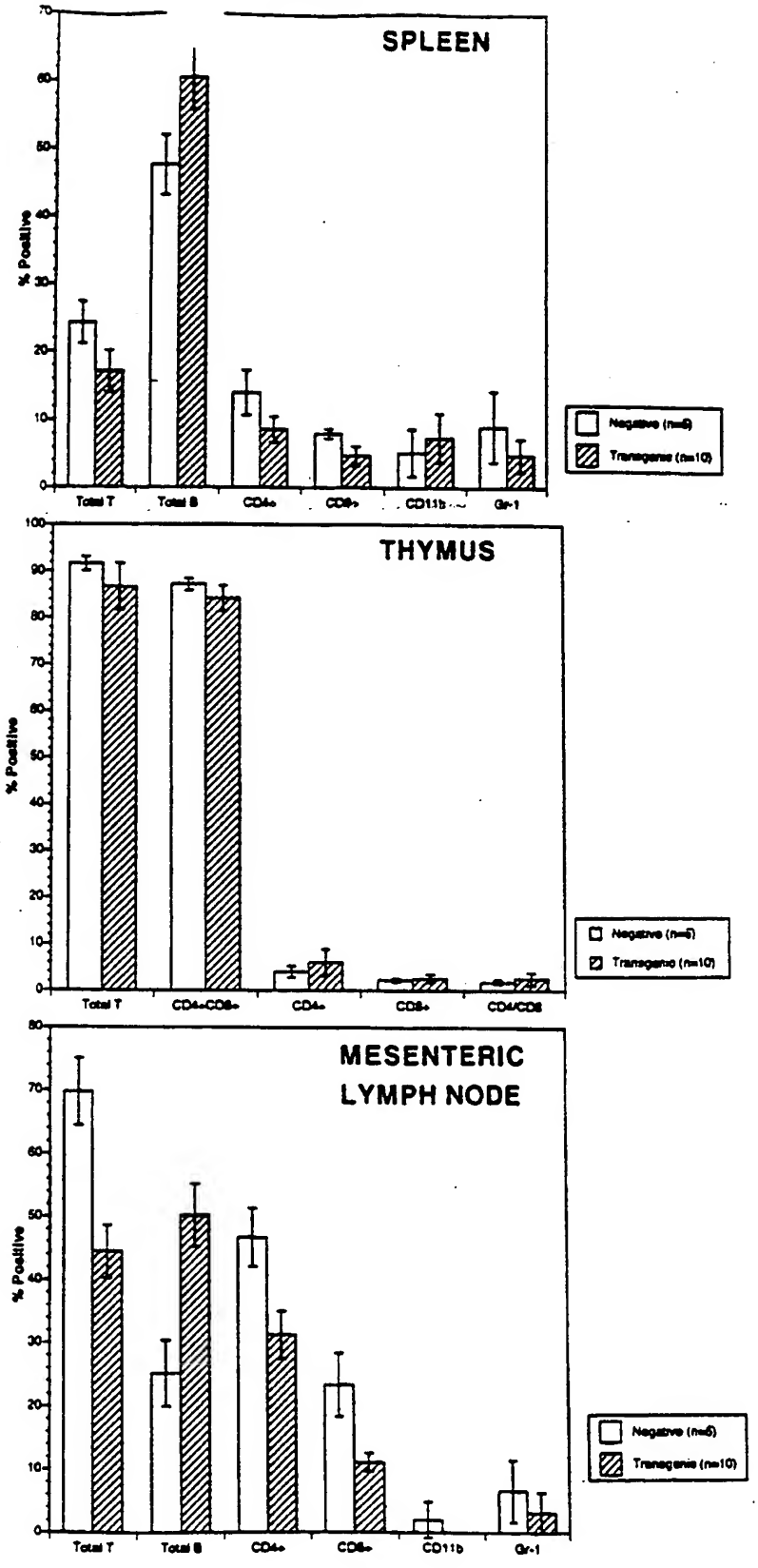

SPLEEN
% Positive
Total T
Total B
CD4+
CD8+
CD11b
Gr-1
Negative (n=6)
Transgene (n=10)
THYMUS
% Positive
Total T
CD4+CD8+
CD4+
CD8+
CD4/CD8
Negative (n=6)
Transgene (n=10)
MESENTERIC LYMPH NODE
% Positive
Total T
Total B
CD4+
CD8+
CD11b
Gr-1
Negative (n=6)
Transgene (n=10)

FIGURE 9

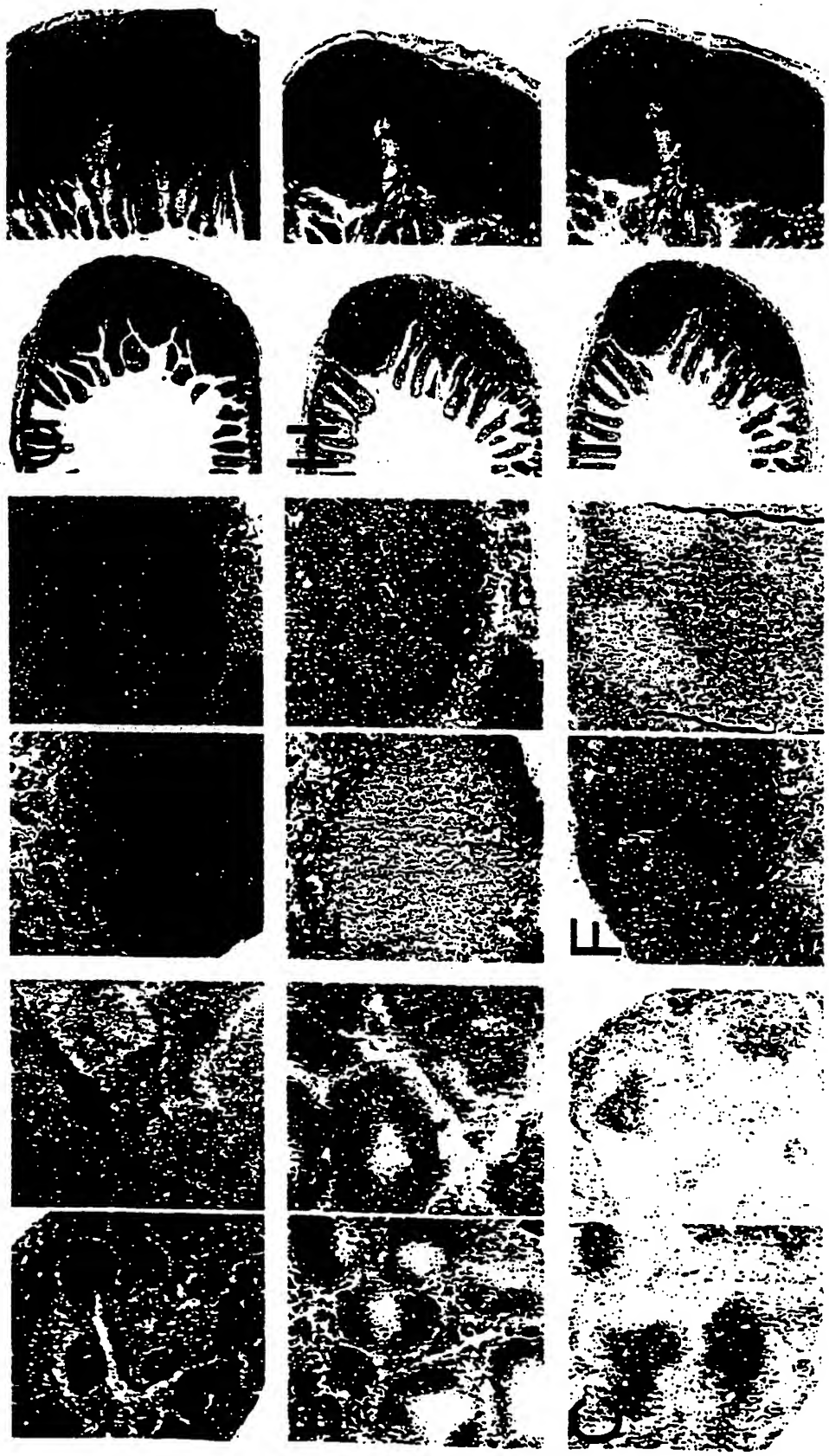
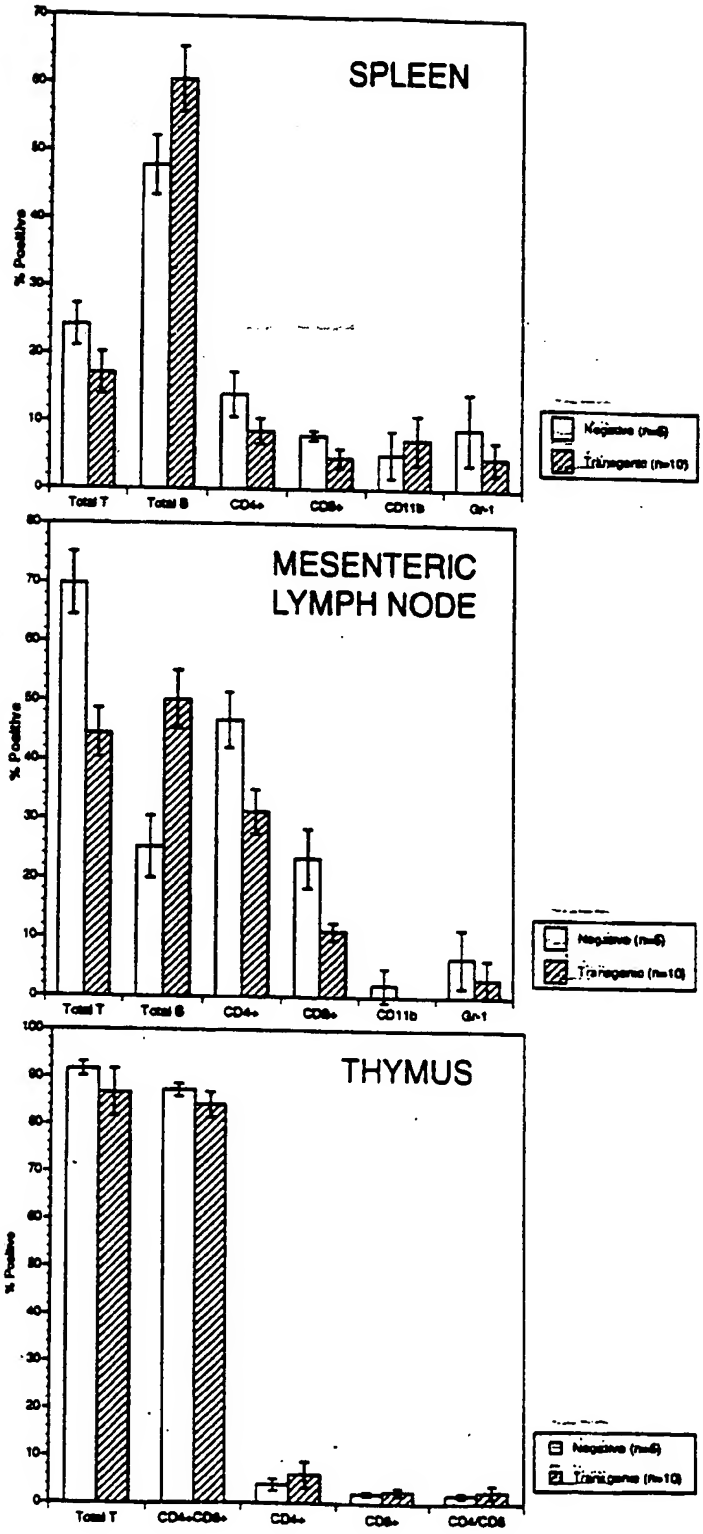


FIGURE 10

FIGURE 11


SPLEEN
% Positive
Total T
Total B
CD4+
CD8+
CD11b
Gr-1
MESENTERIC LYMPH NODE
% Positive
Total T
Total B
CD4+
CD8+
CD11b
Gr-1
THYMUS
% Positive
Total T
CD4+CD8+
CD4+
CD8+
CD4/CD8

IgA (μg/ml)
IgM (μg/ml)
IgE (ng/ml)
IgG (μg/ml)
Age of mice (weeks)
control
transgenic

FIGURE 12

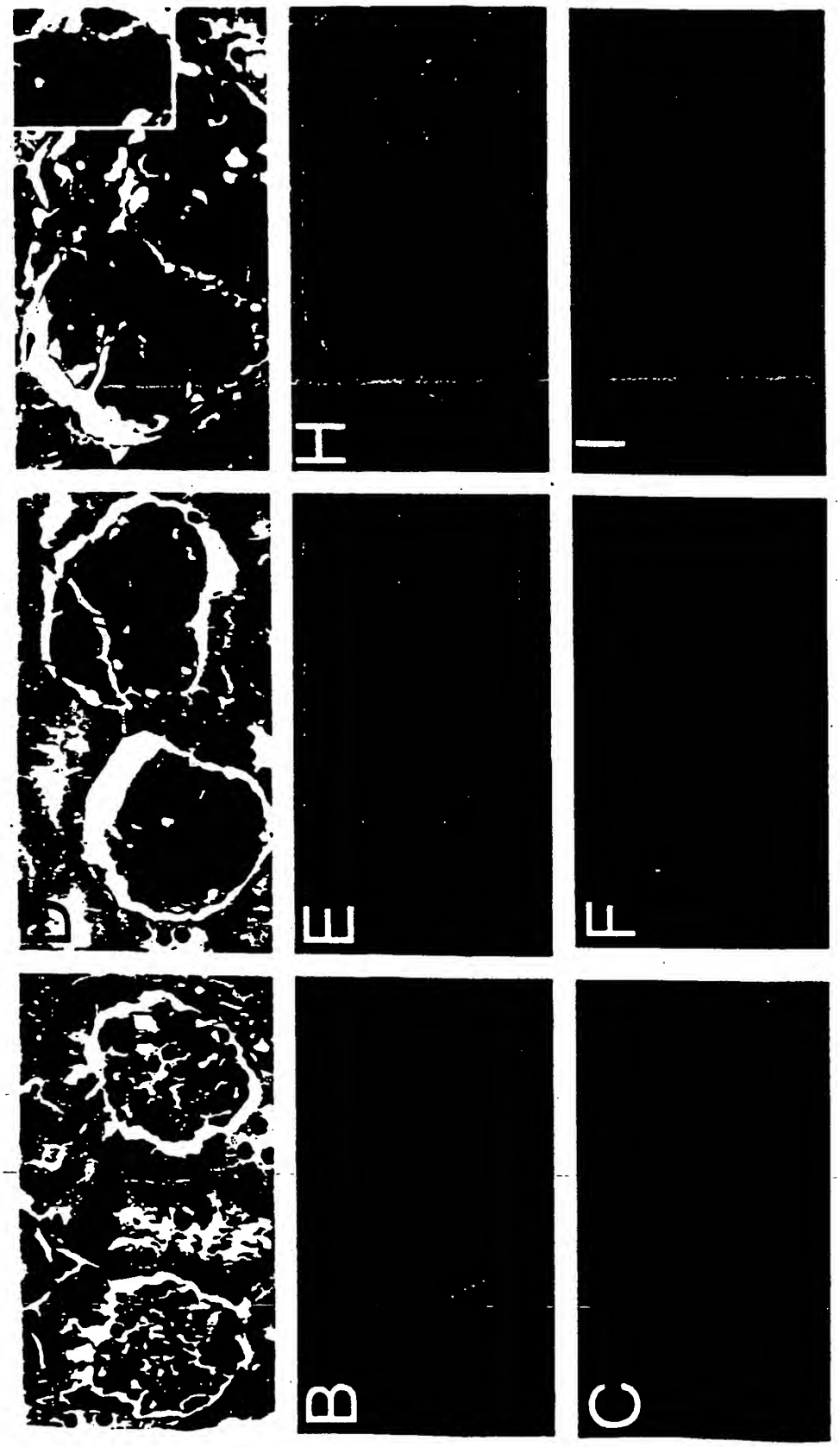
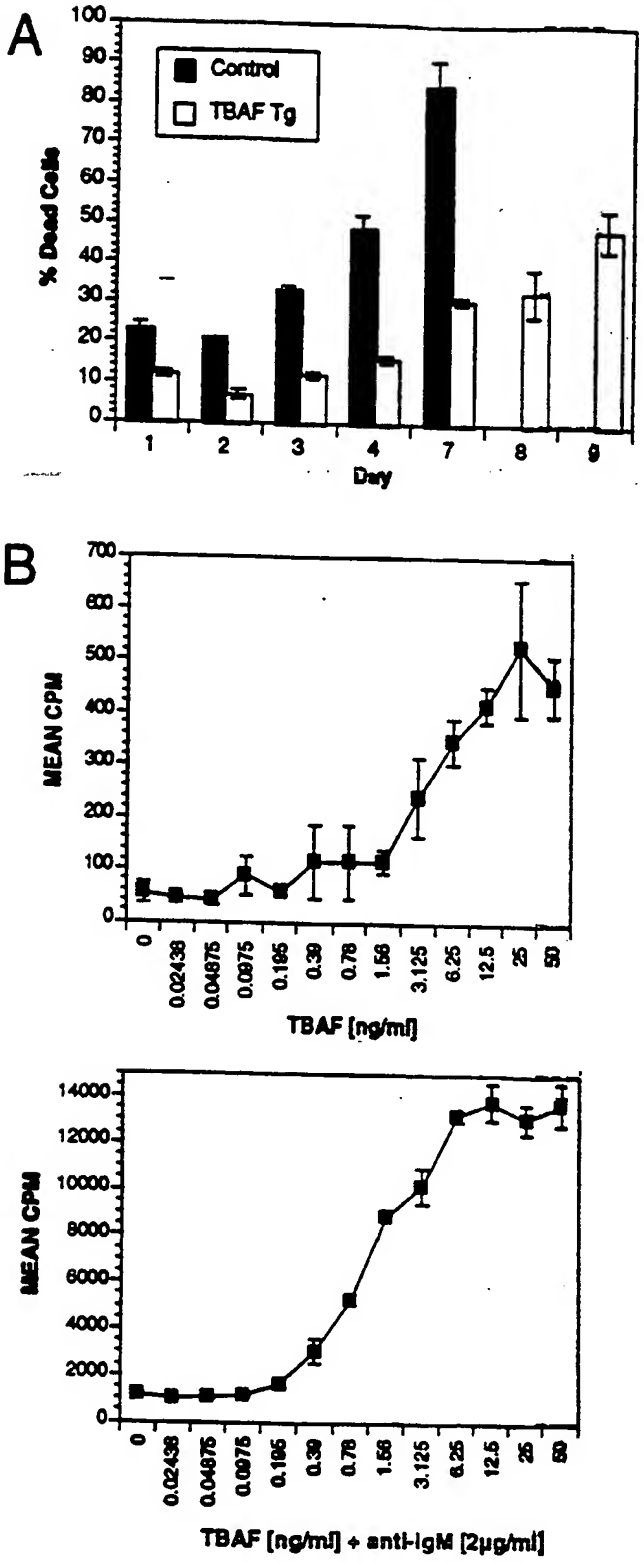


FIGURE 13

FIGURE 14


A
Control
TBAF Tg
% Dead Cells
Day
B
MEAN CPM
TBAF [ng/ml]
MEAN CPM
TBAF [ng/ml] + anti-IgM [2µg/ml]

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(74) Agents: ODRE, Steven et al.; Amgen, Inc., One Amgen Center Drive, Thousand Oaks, CA 91320-1799 (US).

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(54) Title: TNF-RELATED PROTEINS

(57) Abstract: A member of the tumor necrosis factor family and associated antibodies and uses are described. This member is primarily expressed in B cells and its expression correlates to increases in the number of B cells and immunoglobulins produced. The human ortholog contains 285 amino acids; the mouse ortholog, 309 amino acids. The protein is a type II transmembrane protein and has an amino terminal cytoplasmic domain, a transmembrane domain, and a carboxy terminal extracellular domain. TNF-related proteins of the invention may be membrane-associated or in soluble form, recombinantly produced or isolated after natural production. The invention provides for nucleic acids encoding such TNF-related proteins, vectors and host cells expressing the polypeptides, and methods for producting recombinant porteins. Antibodies, fragments, and related fusion proteins and derivatives may be used as agonists or antagonists of AGP-3 related activity.

TNF-RELATED PROTEINS

Cross-reference to Related Applications

This specification is related to U.S. provisional application nos. 60/119,906, filed February 12, 1999 and 60/166,271, filed November 18, 1999, respectively, both of which are hereby incorporated by reference in their entirety.

Field of the Invention

The present invention relates to proteins that are involved in inflammation and immunomodulation, particularly in B cell growth, survival, or activation. The invention further relates to proteins related to the tumor necrosis factor (TNF)/nerve growth factor (NGF) superfamily and related nucleic acids, expression vectors, host cells, and binding assays. The specification also describes compositions and methods for the treatment of immune-related and inflammatory, autoimmune and other immune-related diseases or disorders, such as rheumatoid arthritis (RA), Crohn's disease (CD), lupus, and graft versus host disease (GvHD).

The invention also relates to methods and compositions for the treatment of inflammatory and immune-related diseases and disorders using the receptors.

Background of the Invention

After years of study in necrosis of tumors, tumor necrosis factors (TNFs) α and β were finally cloned in 1984. The ensuing years witnessed the emergence of a superfamily of TNF cytokines, including fas ligand (FasL), CD27 ligand (CD27L), CD30 ligand (CD30L), CD40 ligand (CD40L), TNF-related apoptosis-inducing ligand (TRAIL, also designated AGP-1), osteoprotegerin binding protein (OPG-BP or OPG ligand), 4-1BB ligand, LIGHT, APRIL, and TALL-1. Smith et al. (1994), Cell **76**: 959-962; Lacey et al. (1998), Cell **93**: 165-176; Chichepotiche et al. (1997), J. Biol.

Chem. 272: 32401-32410; Mauri et al. (1998), Immunity 8: 21-30; Hahne et al. (1998), J.Exp. Med. 188: 1185-90; Shu et al. (1999), J. Leukocyte Biology 65: 680-3. This family is unified by its structure, particularly at the C-terminus. In addition, most members known to date are expressed in immune compartments, although some members are also expressed in other tissues or organs, as well. Smith et al. (1994), Cell 76: 959-62. All ligand members, with the exception of LT-α, are type II transmembrane proteins, characterized by a conserved 150 amino acid region within the C-terminal extracellular domain. Though restricted to only 20-25% identity, the conserved 150 amino acid domain folds into a characteristic β-pleated sheet sandwich and trimerizes. This conserved region can be proteolyticaly released, thus generating a soluble functional form. Banner et al. (1993), Cell 73: 431-445.

Many members within this ligand family are expressed in lymphoid enriched tissues and play important roles in immune system development and modulation. Smith et al. (1994). For example, TNFα is mainly synthesized by macrophages and is an important mediator for inflammatory responses and immune defenses. Tracey & Cerami (1994), Annu. Rev. Med. 45: 491-503. Fas-L, predominantly expressed in activated T cell, modulates TCR-mediated apoptosis of thymocyts. Nagata, S. & Suda, T. (1995) Immunology Today 16: 39-43; Castrim et al. (1996), Immunity 5: 617-27. CD40L, also expressed by activated T cells, provides an essential signal for B cell survival, proliferation and immunoglobulin isotype switching. Noelle (1996), Immunity 4: 415-9.

The cognate receptors for most of the TNF ligand family members have been identified. These receptors share characteristic multiple cysteine-rich repeats within their extracellular domains, and do not possess catalytic motifs within cytoplasmic regions. Smith et al. (1994). The receptors signal through direct interactions with death domain proteins (e.g. TRADD, FADD, and RIP) or with the TRAF proteins (e.g.

TRAF2, TRAF3, TRAF5, and TRAF6), triggering divergent and overlapping signaling pathways, e.g. apoptosis, NF-κB activation, or JNK activation. Wallach et al. (1999), Annual Review of Immunology 17: 331-67. These signaling events lead to cell death, proliferation, activation or differentiation. The expression profile of each receptor member varies. For example, TNFR1 is expressed on a broad spectrum of tissues and cells; whereas the cell surface receptor of OPGL is mainly restricted to the osteoclasts. Hsu et al. (1999) Proc. Natl. Acad. Sci. USA 96: 3540-5. It is therefore an object of the invention to identify proteins and nucleic acids related to TNFs. Such proteins are believed to play a role in inflammatory and immune processes, suggesting their usefulness in treating autoimmune and inflammatory disorders.

Summary of the Invention

In accordance with the present invention, the inventors describe a novel member of the tumor necrosis factor family. The novel TNF ligand family member is herein called AGP-3. Unlike other members of the family, the receptor for AGP-3 is primarily expressed in B cells, and its expression correlates to increases in the number of B cells and immunoglobulins produced.

The natural, preferred human ortholog is here called hAGP-3 and contains 285 amino acids; the mouse ortholog (mAGP-3), contains 309 amino acids. The AGP-3 protein is a type II transmembrane protein and has an amino terminal cytoplasmic domain, a transmembrane domain, and a carboxy terminal extracellular domain. TNF-related proteins of the invention may be membrane-associated or in soluble form, recombinantly produced or isolated after natural production. The present specification demonstrates that AGP-3 is a potent B cell stimulatory factor. Interestingly, the AGP-3 transgenic mice also developed autoantibodies

and kidney immune complex deposits, a phenotype resembling lupus patients and lupus prone mice.

The invention provides for nucleic acids encoding AGP-3, vectors and host cells expressing the polypeptides, and methods for producing recombinant proteins. Antibodies or fragments thereof that specifically bind AGP-3 are also provided.

The subject proteins may be used in assays to identify cells and tissues that express AGP-3 or proteins related to AGP-3 and to identify new AGP-3-related proteins. Methods of identifying compounds that interact with AGP-3 proteins are also provided. Such compounds include nucleic acids, peptides, proteins, carbohydrates, lipids or small molecular weight organic molecules and may act either as agonists or antagonists of AGP-3 or AGP-3 R-protein activity.

AGP-3-related proteins are involved in B cell growth, survival, and activation, particularly in lymph node, spleen, and Peyer's patches. AGP-3 agonists and antagonists (e.g., antibodies to AGP-3) thus modulate B cell response and may be used to treat diseases characterized by inflammatory processes or deregulated immune response, such as RA, GvHD, CD, lupus, and the like. Pharmaceutical compositions comprising AGP-3-related proteins and AGP-3 agonists and antagonists are also encompassed by the invention.

In addition to therapeutic applications, AGP-3 related proteins may also be useful in production of hybridoma cells, which are derived from B cells. Thus, the present invention also concerns a method to modulate hybridoma cell antibody production, which comprises treating hybridoma cells with the compound of Claim 1.

Description of the Figures

Figure 1 shows the sequence of human AGP-3. Nucleic acid and amino acid sequences of human AGP-3 are indicated (SEQ ID NOS: 1 and

2, respectively). The predicted transmembrane region is underlined. Potential N-linked glycosylation sites are shown in boldface.

Figure 2 shows the sequence of murine AGP-3. Nucleic acid and amino acid sequences of murine AGP-3 are indicated (SEQ ID NOS: 3 and 4, respectively). The predicted transmembrane region is underlined. Potential N-linked glycosylation sites are shown in boldface.

Figure 3 shows an alignment of human and murine AGP-3, along with a consensus sequence (SEQ ID NO: 5). The predicted human and murine AGP-3 protein sequences were aligned by Pileup with gap creation penalty (12) and gap extension penalty (4) (Wisconsin GCG Package, Version 8.1, Genetics Computer Group Inc., Madison, Wisconsin). The consensus sequence was determined by Lineup (Wisconsin GCG Package, Version 8.1). The transmembrane regions from amino acid 47 to 72 in human AGP-3 and from amino acid 48 to 73 in murine AGP-3 are underlined. The N-terminal intracellular domain resides from amino acid 1 to 46 in human AGP-3 and from amino acid 1 to 47 in murine AGP-3. The C-terminal extracellular domain is localized from amino acid 73 to 285 in human AGP-3, and from amino acid 74 to 309. The human and murine AGP-3 share 68% amino acid identity overall. The C-terminus of AGP-3 is more conserved between human and mouse, with 87% identity over a 142-amino acid length. The putative conserved beta strands are indicated at the top, with the amino acids forming the putative strands underlined.

Figure 4 shows human and murine AGP-3 mRNA tissue distribution. Human tissue northern blots (A) and murine tissue northern blots (B) were probed with ^{32}P-labeled human AGP-3 probe (A) or murine AGP-3 probe. The probed blots were exposed to Kodak film for 18 hours (A) or seven days (B).

Figure 5 shows histology analysis of AGP-3 transgenic mouse spleen. The spleen sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and exosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The spleen of the transgenic mouse was enlarged, mainly due to the increase of size and number of the follicles. The B cell staining areas in the spleen follicles in the transgenic mouse were enlarged. The T cell number was slightly diminished.

Figure 6 shows histology analysis of AGP-3 transgenic mouse lymph nodes. The lymph node sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and exosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The lymph node size of the transgenic mouse was enlarged. The B cell number was greatly increased in the transgenic mouse. Instead of restricted to marginal zones of the follicles as in the control mouse, the B cells also filled out the follicular area in the lymph nodes of the transgenic mouse. The T cell number was decreased in the transgenic mouse as compared to the control.

Figure 7 shows histology analysis of AGP-3 transgenic mouse Peyer's patches. The Peyer's patches sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and exosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The histologic and immunohistologic changes were similar to the changes in the lymph node of the transgenic mouse.

Figure 8 shows FACS analysis of thymocytes, splenocytes and lymph node cells from AGP-3 transgenic mouse. Single-cell suspensions were prepared from spleen, lymph nodes and thymus from 10 AGP-3 transgenic mice and 5 control littermates. Cells were stained with FITC or PE-conjugated monoclonal antibodies against Thy-1.2, B220, CD11b, Gr-1,

CD4 or CD8. The B cell population increased by 100% in the transgenic mice as compared to the control mice. The T cell population decreased approximately 36%, with similar reductions in both CD4+ and CD8+ populations. Similar changes, though to a lesser degree, were observed in splenocytes. No differences in thymocyte staining were observed between the transgenic or control group.

Figure 9 shows a sequence comparison of the C-terminal region of members of the TNF ligand family determined via Pileup (Wisconsin GCG Package, Version 8.1). Amino acid numbers are indicated on the left side. The putative conserved beta strands and loops are indicated at the top. The predicted N-glycosylation sites are indicated with asterisks. The top line shows the consensus sequence (SEQ ID NO: 6). The remaining lines show the sequence for the C-terminal region of the mammalian TNF-related protein identified (SEQ ID NOS: 7 to 24, 40)

Figure 10 shows histology analysis of AGP-3 transgenic mice. Sections of spleen (A, B, C), lymph node (D, E, F) and Payer's patches (G, H, I) from control mice (left panel) and AGP-3 transgenic mice (right panel) were stained with hematoxylin and exosin (A, D, and G), anti-mouse B220 antibody (B, E, and H), or anti-mouse CD3 antibody (C, F, and I). Stained sections were analyzed under microscope at 10x.

Figure 11 shows FACS analysis splenocytes, lymph node cells and thymocytes of AGP-3 transgenic mice. Single-cell suspensions were prepared form spleen, lymph nodes and thymus from 10 AGP-3 transgenic mice and 5 control littermates. Cells with stained with FITC or PE-conjugated monoclonal antibodies against thy-1.2, B220, CD11b, Gr-1, CD4 or CD8.

Figure 12 shows elevation of serum immunoglobulin levels in AGP-3 transgenic mice. Control mice (n=5) and AGP-3 transgenic mice (n=5) were bled successively at 6, 7, 8, 9, 11 and 12 weeks of age. Serum IgM,

IgG, IgA, and IgE levels were quantitated by ELISA. Values are expressed as Mean ± SEM. All AGP-3 immunoglobulin levels were significantly increased (T-test; $P < 0.05$) compared to control groups.

Figure 13 shows kidney immunoglobulin deposits in AGP-3 transgenic mice. Kidney sections of 5 month control littermate (A, B, C), 5 month old AGP-3 mice (D, E, F), and 8 month old AGP-3 mice (G, H, I) were stained hematoxylin and exosin (A, D, and G), anti-mouse IgM (B, E, and H), anti-mouse IgG (C, F, and I), and Trichrome (G insert) Stained sections were analyzed under microscope at 60x.

Figure 14 shows that AGP-3 stimulates B cell survival and proliferation.

A. Increased B cell viability in AGP-3 transgenic mice. B cells were isolated from spleens of 3 month old AGP-3 transgenic mice (n-3) and control littermates (n=3). A total of $2.5x10^5$ B cells was aliquoted per well in a 96-well round bottom plate and incubated for 9 days. At the indicated days, cells were incubated with 5 µg/ml Propidium Iodide and subject to FACS analysis for positive staining cells. Values are expressed as Mean ± SEM.

B. AGP-3 stimulates B cell proliferation. Purified B cells (10^5) from B6 mice were cultured in triplicates in 96 well plate with indicated amount of AGP-3 at the absence (upper panel) or presence of 2 µg/ml anti-IgM antibody (lower panel) for a period of 4 days. Proliferation was measured by radioactive ^{3}(H) thymidine uptake in last 18 hours of pulse. Data shown represent mean ± standard deviation of triplicate wells.

Detailed Description of the Invention

Definition of Terms

The following definitions apply to the terms used throughout this specification, unless otherwise limited in specific instances.

The term "AGP-3 related protein" refers to natural and recombinant proteins comprising the following sequence:

QDCLQLIADSXTPTIXKGXYTFVPWLLSF

(SEQ ID NO: 25)

wherein "X" may be any naturally occurring amino acid residue. This sequence is a consensus of the B and B' β-sheets and B/B' loop of hAGP-3 and mAGP-3 (see Figure 3), which is believed to be the specific receptor binding site. Preferred AGP-3-related proteins comprise both the B/B' consensus and the E/F consensus:

AMGHXIQRKKVHVFGDELSLVTLFR

(SEQ ID NO: 26)

The E/F region is also believed to be involved in receptor binding. More preferred proteins are those comprising the consensus of the B-I region:

QDCLQLIADS XTPTIXKGXY TFVPWLLSFK RGXALEEKEN KIXVXXTGYF FIYXQVLYTD XXXAMGHXIQ RKKVHVFGDE LSLVTLFRCI QNMPXTLPNN SCYSAGIAXL EEGDEXQLAI PRENAQISXX GDXTFFGALK LL

(SEQ ID NO: 27)

"AGP-3-related activity" means that a natural or recombinant protein, analog, derivative or fragment is capable of modulating B cell growth, survival, or activation, particularly in MLN, spleen, and Peyer's patches. The inventors contemplate that some molecules of interest may have activity antagonistic to native AGP-3 activity; for example, a derivative or analog may retain AGP-3 binding activity but will not activate the AGP-3 receptor. All such activity (agonism and antagonism of AGP-3) falls within the meaning of "AGP-3 related activity." Such activity can be determined, for example, by such assays as described in "Biological activity of AGP-3" in the Materials & Methods hereinafter, which may be modified as needed by many methods known to persons having ordinary skill in the art.

An "analog" of an AGP-3 protein (e.g., hAGP-3) is a polypeptide within the definition of "AGP-3-related protein" or "AGP-3-related protein," respectively, that has a substitution or addition of one or more amino acids. Such an AGP-3-related protein should maintain the property of eliciting B cell growth, survival, or activation. Such analogs will have substitutions or additions at any place along the polypeptide. Preferred analogs include those of soluble AGP-3-related proteins. Fragments or analogs may be naturally occurring, such as a polypeptide product of an allelic variant or a mRNA splice variant, or they may be constructed using techniques available to one skilled in the art for manipulating and synthesizing nucleic acids. The polypeptides may or may not have an amino terminal methionine residue.

A "derivative" of an AGP-3 protein is a polypeptide within the definition of "AGP-3-related protein" that has undergone post-translational modifications. Such modifications include, for example, addition of N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends, attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of an N-terminal methionine residue due to prokaryotic host cell expression. In particular, chemically modified derivatives of AGP-3-related protein that provide additional advantages such as increased stability, longer circulating time, or decreased immunogenicity are contemplated. Of particular use is modification with water soluble polymers, such as polyethylene glycol and derivatives thereof (see for example U.S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule,

or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties. Polypeptides may also be modified at pre-determined positions in the polypeptide, such as at the amino terminus, or at a selected lysine or arginine residue within the polypeptide. Other chemical modifications provided include a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

The term "protein" refers to polypeptides regardless of length or origin, comprising molecules that are recombinantly produced or naturally occurring, full length or truncated, having a natural sequence or mutated sequence, with or without post-translational modification, whether produced in mammalian cells, bacterial cells, or any other expression system.

The invention provides for proteins referred to as AGP-3 protein, or AGP-3-related proteins that primarily act on B cells. An EST bearing a portion of the AGP-3 sequence was obtained from a human fetal liver spleen cDNA library. A labeled cDNA fragment was used to probe a human spleen cDNA phage library (see "Cloning of Human AGP-3" in Materials & Methods hereinafter). The cDNA encoding a human AGP-3 was isolated from this phage library. The human protein is a type II transmembrane protein, having a short N-terminal intracellular region that differed from other members of the TNF ligand family and a long C-terminal extracellular region that comprises most of the conserved region of the TNF ligand family.

An EST encoding a murine ortholog was identified by BLAST search of Genebank using the human AGP-3 sequence. The corresponding cDNA clone was obtained from a mouse lymph node library and used to probe a mouse spleen cDNA phage library (see Materials & Methods

hereinafter). The cDNA encoding a murine AGP-3 ortholog was isolated from this phage library.

Northern blots were used to determine tissue distribution of transcription of AGP-3 (see "Cloning of Murine AGP-3" in Materials & Methods hereinafter). In murine tissue, AGP-3 mRNA was detected mainly in spleen, lung, liver, and kidney. In human tissue, AGP-3 mRNA was detected predominantly in peripheral blood leukocytes, with weaker transcription in spleen, lung, and small intestine (see Figures 4A and 4B).

The murine ortholog of AGP-3 was overexpressed in transgenic mice (see "Overexpression of murine AGP-3 in transgenic mice" in Materials & Methods hereinafter). In these transgenic mice, serum globulin and total protein levels increased greatly over control littermates while the albumin level remained the same (see "Biological Activity of AGP-3" in Materials & Methods hereinafter). The mice also exhibited increases in the size and number of follicles in the spleen, lymph nodes, and Peyer's patches (Figures 5, 6, and 7). In their MLN, the mice exhibited 100% increases in the number of cells expressing CD45 receptor with concomitant decreases in cells expressing CD90, CD4, and CD8. These results correspond to an increase in the B cell population and a decrease in the T cell population in the MLN (Figures 6 and 8). Similar results were obtained in the spleen, but to a lesser extent (Figures 5 and 8).

Nucleic Acids

The invention provides for isolated nucleic acids encoding AGP-3-related proteins. As used herein, the term "nucleic acid" comprises cDNA, genomic DNA, wholly or partially synthetic DNA, and RNA. These nucleic acids may be prepared or isolated as described in the working examples hereinafter or by nucleic acid hybridization thereof.

Nucleic acid hybridization typically involves a multi-step process. A first hybridization step forms nucleic acid duplexes from single strands.

A second hybridization step under more stringent conditions selectively retains nucleic acid duplexes having the desired homology. The conditions of the first hybridization step are generally not crucial, provided they are not of higher stringency than the second hybridization step. Generally, the second hybridization is carried out under conditions of high stringency, wherein "high stringency" conditions refers to conditions of temperature and salt that are about 12-20 °C below the melting temperature (T_m) of a perfect hybrid of part or all of the complementary strands corresponding to Figure 1 (SEQ ID NO: 1) and Figure 2 (SEQ ID NO: 3). In one embodiment, "high stringency" conditions refer to conditions of about 65 °C and not more than about 1 M Na^+. It is understood that salt concentration, temperature and/or length of incubation may be varied in either the first or second hybridization steps such that one obtains the hybridizing nucleic acid molecules according to the invention. Conditions for hybridization of nucleic acids and calculations of T_m for nucleic acid hybrids are described in Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, New York.

The nucleic acids of the invention may hybridize to part or all of the polypeptide coding regions of AGP-3 related proteins (e.g., SEQ ID NOS: 2 and 4 as shown in Figures 1 and 2) and therefore may be truncations or extensions of the nucleic acid sequences shown therein. Truncated or extended nucleic acids are encompassed by the invention provided that the encoded proteins retain AGP-3 related activity. In one embodiment, the nucleic acid will encode a polypeptide of at least about 10 amino acids. In another embodiment, the nucleic acid will encode a polypeptide of at least about 20 amino acids. In yet another embodiment, the nucleic acid will encode a polypeptide of at least about 50 amino acids. The hybridizing nucleic acids may also include noncoding sequences located 5' and/or 3' to the coding regions for the AGP-3 related protein. Noncoding

sequences include regulatory regions involved in expression of AGP-3 related protein, such as promoters, enhancer regions, translational initiation sites, transcription termination sites and the like.

In preferred embodiments, the nucleic acids of the invention encode mouse or human AGP-3. Most preferred are the nucleic acids encoding proteins of SEQ ID NOS: 25, 26, or 27. Nucleic acids may encode a membrane-bound form of AGP-3-related protein or soluble forms. For human AGP-3-related protein, the predicted transmembrane region includes amino acid residues 47-72 inclusive as shown in Figure 1 (SEQ. ID. NO: 2); for murine AGP-3 related protein, residues 48-73 inclusive as shown in Figure 2 (SEQ ID NO: 4). Substitutions that replace hydrophobic amino acid residues in this region with neutral or hydrophilic amino acid residues would be expected to disrupt membrane association and result in soluble AGP-3-related protein. In addition, deletions of part or all the transmembrane region would also be expected to produce soluble forms of AGP-3-related protein. Nucleic acids encoding SEQ ID NO: 5 as shown in Figure 3 or fragments and analogs thereof, encompass soluble AGP-3-related proteins.

Nucleic acid sequences of the invention may also be used for the detection of sequences encoding AGP-3-related protein in biological samples. In particular, the sequences may be used to screen cDNA and genomic libraries for related AGP-3-related protein sequences, especially those from other species. The nucleic acids are also useful for modulating levels of AGP-3-related protein by anti-sense technology or in vivo gene expression. Development of transgenic animals expressing AGP-3-related protein are useful for production of the polypeptides and for the study of in vivo biological activity.

Vectors and Host Cells

The nucleic acids of the invention will be linked with DNA sequences so as to express biologically active AGP-3-related protein. Sequences required for expression are known to those skilled in the art and include promoters and enhancer sequences for initiation of RNA synthesis, transcription termination sites, ribosome binding sites for the initiation of protein synthesis, and leader sequences for secretion. Sequences directing expression and secretion of AGP-3-related protein may be homologous, i.e., the sequences are identical or similar to those sequences in the genome involved in AGP-3-related protein expression and secretion, or they may be heterologous. A variety of plasmid vectors are available for expressing AGP-3-related protein in host cells (see, for example, Methods in Enzymology v. 185, Goeddel, D.V. ed., Academic Press (1990)). For expression in mammalian host cells, a preferred embodiment is plasmid pDSRα described in PCT Application No. 90/14363. For expression in bacterial host cells, preferred embodiments include plasmids harboring the lux promoter (see co-owned and co-pending U.S. Serial No. 08/577,778, filed December 22, 1995). In addition, vectors are available for the tissue-specific expression of AGP-3-related protein in transgenic animals. Gene transfer vectors derived from retrovirus (RV), adenovirus (AdV), and adeno-associated virus (AAV) may also be used for the expression of AGP-3 related protein in human cells for in vivo therapy (see PCT Application No. 86/00922).

Prokaryotic and eukaryotic host cells expressing AGP-3-related protein are also provided by the invention. Host cells include bacterial, yeast, plant, insect or mammalian cells. AGP-3-related protein may also be produced in transgenic animals, such as mice or goats. Plasmids and vectors containing the nucleic acids of the invention are introduced into appropriate host cells using transfection or transformation techniques known to one skilled in the art. Host cells may contain DNA sequences

encoding AGP-3-related protein as shown in Figures 1, 2, or 3, or a portion of either thereof, such as the extracellular domain or the cytoplasmic domain. Nucleic acids encoding AGP-3-related proteins may be modified by substitution of codons that allow for optimal expression in a given host. At least some of the codons may be so-called preference codons that do not alter the amino acid sequence and are frequently found in genes that are highly expressed. However, it is understood that codon alterations to optimize expression are not restricted to the introduction of preference codons. Examples of preferred mammalian host cells for AGP-3-related protein expression include, but are not limited to COS, CHOd-, 293 and 3T3 cells. A preferred bacterial host cell is Escherichia coli.

Polypeptides

The invention also provides AGP-3-related proteins as the products of prokaryotic or eukaryotic expression of exogenous DNA sequences. Exogenous DNA sequences include cDNA, genomic DNA and synthetic DNA sequences. AGP-3-related proteins may be the products of bacterial, yeast, plant, insect or mammalian cells expression, or from cell-free translation systems. AGP-3-related proteins produced in bacterial cells will have N-terminal methionine residues. The invention also provides for a process of producing AGP-3-related proteins comprising growing prokaryotic or eukaryotic host cells transformed or transfected with nucleic acids encoding them and isolating polypeptide expression products of the nucleic acids.

Polypeptides that are mammalian proteins or are fragments, analogs or derivatives thereof are encompassed by the invention. In preferred embodiments, the AGP-3-related protein is human AGP-3 protein. A fragment of AGP-3-related protein refers to a polypeptide having a deletion of one or more amino acids such that the resulting polypeptide retains AGP-3 related activity; for example, the polypeptide

has at least the property of eliciting or antagonizing B cell growth, survival, or activation, especially in mesenteric lymph nodes. Said fragments will have deletions originating from the amino terminal end, the carboxy terminal end, and internal regions of the polypeptide. Fragments of AGP-3-related proteins are at least about ten amino acids, at least about 20 amino acids, or at least about 50 amino acids in length. In preferred embodiments, AGP-3-related proteins will have a deletion of one or more amino acids from the transmembrane region (amino acid residues 48-73 as shown in Figure 1), or, alternatively, one or more amino acids from the amino-terminus up to and/or including the transmembrane region (amino acid residues 1-73 as shown in Figure 1). Such polypeptides may act as agonists or antagonists of the ligand:receptor interaction and activate or inhibit ligand-mediated activity of AGP-3 related protein. Such antagonists and/or agonists can be examined for AGP-3 related activity (see "Biological activity of AGP-3" in Materials & Methods hereinafter).

The polypeptides of the invention are isolated and purified from tissues and cell lines that express AGP-3 related protein, either extracted from lysates or from conditioned growth medium, and from transformed host cells expressing AGP-3 related protein. Human AGP-3 related protein, or nucleic acids encoding same, may be isolated from human lymph node or fetal liver tissue. Isolated AGP-3 related protein is free from association with human proteins and other cell constituents.

A method for purification of such proteins from natural sources (e.g. tissues and cell lines that normally express an AGP-3 related protein) and from transfected host cells is also encompassed by the invention. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The chromatography steps can include ion exchange, gel filtration,

hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-AGP-3-related protein antibody or biotin-streptavidin affinity complex and the like.

Fusion proteins and derivatives

The invention further comprises AGP-3-related protein chimeras, as well as such proteins derivatized by linkage to such molecules as PEG or dextran. Such proteins comprise part or all of an AGP-3-related protein amino acid sequence fused to a heterologous amino acid sequence. The heterologous sequence may be any sequence that allows the resulting fusion protein to retain AGP-3-related activity (i.e., AGP-3 agonists) or will maintain AGP-3 binding activity but not have AGP-3 related activity as defined herein (i.e., AGP-3 antagonists). Such fragments, derivatives or analogs of AGP-3 can be examined for their ability to agonize or antagonize AGP-3-mediated B cell growth, survival, or activation associated with the disease or condition (see "Biological activity of AGP-3" in Materials & Methods hereinafter). In preferred embodiments, a heterologous sequence is fused to a sequence comprising an AGP-3 related protein's B/B' region (SEQ ID NO: 25) and/or the E/F region (SEQ ID NO: 26) or to the more complete B-I region (SEQ ID NO: 27). Such heterologous sequences include cytoplasmic domains that allow for alternative intracellular signaling events, sequences that promote oligomerization (e.g., the Fc region of IgG), enzyme sequences that provide a label for the polypeptide, and sequences that provide affinity probes (e.g., an antigen-antibody recognition site).

Preferred molecules in accordance with this invention are Fc-linked AGP-3 related proteins. Useful modifications of protein therapeutic agents by fusion with the "Fc" domain of an antibody are discussed in detail in a patent application entitled, "Modified Peptides as Therapeutic Agents," U.S. Ser. No. 09/428,082, PCT appl. no. WO 99/25044, which is hereby

incorporated by reference in its entirety. That patent application discusses linkage to a "vehicle" such as PEG, dextran, or an Fc region.

In the compositions of matter prepared in accordance with this invention, the AGP-3 related protein may be attached to a vehicle through the protein's N-terminus or C-terminus. Thus, the vehicle-protein molecules of this invention may be described by the following formula I:

I

$$(X^1)_a\text{-}F^1\text{-}(X^2)_b$$

wherein:

F^1 is a vehicle (preferably an Fc domain);

X^1 and X^2 are each independently selected from $-(L^1)_c\text{-}P^1$, $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2$, $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3$, and $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3\text{-}(L^4)_f\text{-}P^4$

P^1, P^2, P^3, and P^4 are each independently sequences of AGP-3 related protein (e.g., a fragment of hAGP-3);

L^1, L^2, L^3, and L^4 are each independently linkers; and

a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1.

Thus, compound I comprises preferred compounds of the formulae

II

$$X^1\text{-}F^1$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the C-terminus of X^1;

III

$$F^1\text{-}X^2$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the N-terminus of X^2;

IV

$$F^1\text{-}(L^1)_c\text{-}P^1$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the N-terminus of $-(L^1)_c-P^1$; and

V

$$F^1-(L^1)_c-P^1-(L^2)_d-P^2$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the N-terminus of $-L^1-P^1-L^2-P^2$.

Antibodies

Uses for antibodies specifically binding the polypeptides of the invention are also encompassed by the invention. The antibodies may be generated by immunization with full-length AGP-3 related protein, or fragments thereof. Preferred antibodies bind to SEQ ID NOS: 25, 26, or 27. Such antibodies may be generated by immunization with polypeptides comprising those sequences. The term "antibodies" also refers to molecules having Fv, Fc and other structural domains usually associated with antibodies but that may be generated by other techniques (e.g., phage display antibody generation). The antibodies of the invention may be polyclonal or monoclonal, or may be recombinant antibodies, such as chimeric antibodies wherein the murine constant regions on light and heavy chains are replaced by human sequences, or CDR-grafted antibodies wherein only the complementarity determining regions are of murine origin. Antibodies of the invention may also be fully human antibodies prepared, for example, by immunization of transgenic animals capable of producing human antibodies (see, for example, PCT Application No. WO93/12227). Regardless of the means by which they are generated, antibodies in accordance with this invention may be produced by recombinant means (e.g., transfection of CHO cells with vectors comprising antibody sequence).

The antibodies are useful for detecting AGP-3 related protein in biological samples, thereby allowing the identification of cells or tissues

that produce such proteins. In addition, antibodies that bind to AGP-3 related proteins and block interaction with other binding compounds (i.e., "antagonist antibodies") have therapeutic use in modulating B cell growth, activation, and/or proliferation. Antibodies can be tested for binding to AGP-3 related protein and examined for their effect on AGP-3-mediated B cell growth, survival, or activation associated with the disease or condition (see "Biological activity of AGP-3" in Materials & Methods hereinafter).

Compositions

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the AGP-3 related protein of the invention together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of an AGP-3 related protein agonist or antagonist. The term "therapeutically effective amount" means an amount that provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form and comprises one or more of the following:

- a diluent (e.g., Tris, acetate or phosphate buffers) having various pH values and ionic strengths;
- a solubilizer (e.g., Tween or Polysorbate);
- carriers (e.g., human serum albumin or gelatin);
- preservatives (e.g., thimerosal or benzyl alcohol); and
- antioxidants (e.g., ascorbic acid or sodium metabisulfite).

Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive survey of component suitable for pharmaceutical compositions is found in

Remington's Pharmaceutical Sciences (1980), 18th ed. (A. R. Gennaro, ed.) Mack, Easton, PA.

In a preferred embodiment, compositions comprising AGP-3 antibody or soluble AGP-3-related protein are provided. Also encompassed are compositions comprising soluble AGP-3-related protein modified with water-soluble polymers to increase solubility, stability, plasma half-life and bioavailability. Compositions may also comprise incorporation of soluble AGP-3 related protein into liposomes, microemulsions, micelles or vesicles for controlled delivery over an extended period of time. Soluble AGP-3 related protein may be formulated into microparticles suitable for pulmonary administration.

Compositions of the invention may be administered by injection (either subcutaneous, intravenous or intramuscular) or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one of ordinary skill in the art.

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the coding region of AGP-3 related protein and/or flanking regions to cells and tissues as part of an anti-sense therapy regimen.

Pharmaceutical Methods of Use

AGP-3 related proteins and agonists or antagonists thereof may be used to treat conditions characterized by B cell growth, survival, and activation, such as autoimmune and inflammatory disorders. The invention also encompasses modulators (agonists and antagonists) of AGP-3-related protein and methods for obtaining them. Such a modulator may either increase or decrease at least one activity associated with AGP-

3, such as B cell growth, survival, or activation in MLN, spleen, and Peyer's patches. Typically, an agonist or antagonist may be a co-factor, such as a protein, peptide, carbohydrate, lipid or small molecular weight molecule, that interacts with AGP-3 and regulates activity. Potential polypeptide antagonists include antibodies that react with soluble or membrane-associated forms of AGP-3, a fragment of AGP-3 (e.g., SEQ ID NO: 25) and an Fc-linked AGP-3 fragment. Molecules that regulate AGP-3-related protein expression typically include nucleic acids that are complementary to nucleic acids encoding AGP-3-related protein and that act as anti-sense regulators of expression.

AGP-3-related proteins and modulators thereof may be particularly useful in treatment of inflammatory conditions of the joints. Inflammatory conditions of a joint are chronic joint diseases that afflict and disable, to varying degrees, millions of people worldwide. Rheumatoid arthritis is a disease of articular joints in which the cartilage and bone are slowly eroded away by a proliferative, invasive connective tissue called pannus, which is derived from the synovial membrane. The disease may involve peri-articular structures such as bursae, tendon sheaths and tendons as well as extra-articular tissues such as the subcutis, cardiovascular system, lungs, spleen, lymph nodes, skeletal muscles, nervous system (central and peripheral) and eyes (Silberberg (1985), Anderson's Pathology, Kissane (ed.), II:1828). Osteoarthritis is a common joint disease characterized by degenerative changes in articular cartilage and reactive proliferation of bone and cartilage around the joint. Osteoarthritis is a cell-mediated active process that may result from the inappropriate response of chondrocytes to catabolic and anabolic stimuli. Changes in some matrix molecules of articular cartilage reportedly occur in early osteoarthritis (Thonar et al. (1993), Rheumatic disease clinics of North America, Moskowitz (ed.), 19:635-657 and Shinmei et al. (1992), Arthritis Rheum.,

35:1304-1308). AGP-3, AGP-3 R and modulators thereof are believed to be useful in the treatment of these and related conditions.

AGP-3 related proteins and agonists or antagonists thereof may also be useful in treatment of a number of additional diseases and disorders, including:

acute pancreatitis;

ALS;

Alzheimer's disease;

asthma;

atherosclerosis;

cachexia/anorexia;

chronic fatigue syndrome;

diabetes (e.g., insulin diabetes);

fever;

glomerulonephritis;

graft versus host disease;

hemorrhagic shock;

hyperalgesia;

inflammatory bowel disease;

inflammatory conditions of a joint, including osteoarthritis, psoriatic arthritis and rheumatoid arthritis;

inflammatory conditions resulting from strain, sprain, cartilage damage, trauma, orthopedic surgery, infection or other disease processes;

ischemic injury, including cerebral ischemia (e.g., brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration);

learning impairment;

lung diseases (e.g., ARDS);

multiple myeloma;

multiple sclerosis;
myelogenous leukemia (e.g., AML and CML) and other leukemias;
myopathies (e.g., muscle protein metabolism, esp. in sepsis);
neurotoxicity (e.g., as induced by HIV);
osteoporosis;
pain;
Parkinson's disease;
pre-term labor;
psoriasis;
reperfusion injury;
septic shock;
side effects from radiation therapy;
sleep disturbance;
temporal mandibular joint disease; and
tumor metastasis.

Agonists and antagonists of AGP-3-related protein may be administered alone or in combination with a therapeutically effective amount of other drugs, including analgesic agents, disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and any immune and/or inflammatory modulators. Thus, agonists and antagonists of AGP-3 related protein may be administered with:

- Modulators of other members of the TNF/TNF receptor family, including TNF antagonists, such as etanercept (Enbrel™), sTNF-RI, D2E7, and Remicade™.
- Nerve growth factor (NGF) modulators.
- IL-1 inhibitors, including IL-1ra molecules such as anakinra (Kineret™) and more recently discovered IL-1ra-like molecules such as IL-1Hy1 and IL-1Hy2; IL-1 "trap" molecules as described

in U.S. Pat. No. 5,844,099, issued December 1, 1998; IL-1 antibodies; solubilized IL-1 receptor, and the like.

- IL-6 inhibitors (e.g., antibodies to IL-6).
- IL-8 inhibitors (e.g., antibodies to IL-8).
- IL-18 inhibitors (e.g., IL-18 binding protein, solubilized IL-18 receptor, or IL-18 antibodies).
- Interleukin-1 converting enzyme (ICE) modulators.
- insulin-like growth factors (IGF-1, IGF-2) and modulators thereof.
- Transforming growth factor-β (TGF-β), TGF-β family members, and TGF-β modulators.
- Fibroblast growth factors FGF-1 to FGF-10, and FGF modulators.
- Osteoprotegerin (OPG), OPG analogues, osteoprotective agents, and bone anabolic agents.
- PAF antagonists.
- Keratinocyte growth factor (KGF), KGF-related molecules (e.g., KGF-2), and KGF modulators.
- COX-2 inhibitors, such as Celebrex™ and Vioxx™.
- Prostaglandin analogs (e.g., E series prostaglandins).
- Matrix metalloproteinase (MMP) modulators.
- Nitric oxide synthase (NOS) modulators, including modulators of inducible NOS.
- Modulators of glucocorticoid receptor.
- Modulators of glutamate receptor.
- Modulators of lipopolysaccharide (LPS) levels.
- Anti-cancer agents, including inhibitors of oncogenes (e.g., fos, jun) and interferons.
- Noradrenaline and modulators and mimetics thereof.

Assay Methods of Use

AGP-3-related proteins may be used in a variety of assays for detecting agonists, antagonists and characterizing interactions with AGP-3 related proteins. In general, the assay comprises incubating AGP-3-related protein under conditions that permit measurement of AGP-3-related activity as defined above. Qualitative or quantitative assays may be developed. Assays may also be used to identify new AGP-3 agonists or antagonists and AGP-3 related potein family members.

Binding assays for agonists, or antagonists to natural or recombinant AGP-3 related protein may be carried out in several formats, including cell-based binding assays, membrane binding assays, solution-phase assays and immunoassays. In general, trace levels of a labeled binding molecule are incubated with AGP-3-related protein samples for a specified period of time followed by measurement of bound molecule by filtration, electrochemiluminescent (ECL, ORIGEN system by IGEN), cell-based or immunoassays. Homogeneous assay technologies for radioactivity (SPA; Amersham) and time-resolved fluoresence (HTRF, Packard) can also be implemented. Binding is detected by labeling a binding molecule (e.g., an anti-AGP-3 antibody) with radioactive isotopes (125I, 35S, 3H), fluorescent dyes (fluorescein), lanthanide (Eu^{3+}) chelates or cryptates, orbipyridyl-ruthenium (Ru^{2+}) complexes. It is understood that the choice of a labeled probe will depend upon the detection system used. Alternatively, a binding molecule may be modified with an unlabeled epitope tag (e.g., biotin, peptides, His_6, myc) and bound to proteins such as streptavidin, anti-peptide or anti-protein antibodies that have a detectable label as described above.

Binding molecules in such assays may be nucleic acids, proteins, peptides, carbohydrates, lipids or small molecular weight organic compounds. The binding molecule may be substantially purified or

present in a crude mixture. The binding molecules may be further characterized by their ability to increase or decrease AGP-3 related activity in order to determine whether they act as an agonist or an antagonist.

In an alternative method, AGP-3-related protein may be assayed directly using polyclonal or monoclonal antibodies to AGP-3 related proteins in an immunoassay. Additional forms of AGP-3-related proteins containing epitope tags as described above may be used in solution and immunoassays.

AGP-3 related proteins are also useful for identification of intracellular proteins that interact with their respective cytoplasmic domains by a yeast two-hybrid screening process. As an example, hybrid constructs comprising DNA encoding the N-terminal 50 amino acids of an AGP-3 related protein fused to a yeast GAL4-DNA binding domain may be used as a two-hybrid bait plasmid. Positive clones emerging from the screening may be characterized further to identify interacting proteins. This information may help elucidate an intracellular signaling mechanism associated with AGP-3-related activity and provide intracellular targets for new drugs that modulate inflammatory and immune-related diseases and conditions.

A variety of assays may be used to measure the interaction of AGP-3-related proteins and agonists, antagonists, or other ligands in vitro using purified proteins. These assays may be used to screen compounds for their ability to increase or decrease the rate or extent of binding to AGP-3 related proteins. In one type of assay, AGP-3 related protein can be immobilized by attachment to the bottom of the wells of a microtiter plate. A radiolabeled binding molecule and a test molecule can then be added either one at a time (in either order) or simultaneously to the wells. After incubation, the wells can be washed and counted using a scintillation counter for radioactivity to determine the extent of binding to AGP-3

related protein. Typically, molecules will be tested over a range of concentrations, and a series of control wells lacking one or more elements of the test assays can be used for accuracy in evaluation of the results. An alternative to this method involves reversing the "positions" of the proteins; i.e., immobilizing a binding molecule to the mictrotiter plate wells, incubating with the test compound and radiolabeled AGP-3 related protein, and determining the extent of binding. See, for example, chapter 18 of Current Protocols in Molecular Biology (1995) (Ausubel et al., eds.), John Wiley & Sons, New York, NY.

As an alternative to radiolabeling, AGP-3 related proteins or a binding molecule may be conjugated to biotin and the presence of biotinylated protein can then be detected using streptavidin linked to an enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), that can be detected colorimetrically, or by fluorescent tagging of streptavidin. An antibody directed to AGP-3 related protein or a binding molecule that is conjugated to biotin may also be used and can be detected after incubation with enzyme-linked streptavidin linked to AP or HRP.

AGP-3-related proteins or binding molecules may also be immobilized by attachment to agarose beads, acrylic beads or other types of such inert substrates. The substrate-protein complex can be placed in a solution containing the complementary protein and the test compound; after incubation, the beads can be precipitated by centrifugation, and the amount of binding between the AGP-3-related protein and a binding molecule can be assessed using the methods described above. Alternatively, the substrate-protein complex can be immobilized in a column and the test molecule and complementary molecule passed over the column. Formation of a complex between AGP-3 related protein and the binding molecule can then be assessed using any of the techniques set forth above (i.e., radiolabeling, antibody binding, and the like).

Another useful in vitro assay is a surface plasmon resonance detector system, such as the Biacore assay system (Pharmacia, Piscataway, NJ). The Biacore system may be carried out using the manufacturer's protocol. This assay essentially involves covalent binding of either an AGP-3 related protein or a binding molecule to a dextran-coated sensor chip that is located in a detector. The test compound and the other complementary protein can then be injected into the chamber containing the sensor chip either simultaneously or sequentially and the amount of complementary protein that binds can be assessed based on the change in molecular mass that is physically associated with the dextran-coated side of the of the sensor chip; the change in molecular mass can be measured by the detector system.

In vitro assays such as those described above may be used advantageously to screen rapidly large numbers of compounds for effects on complex formation with AGP-3-related proteins. The assays may be automated to screen compounds generated in phage display, synthetic peptide and chemical synthesis libraries.

Compounds that increase or decrease complex formation of AGP-3-related proteins and binding molecules may also be screened in cell culture using cells and cell lines bearing such ligands. Cells and cell lines may be obtained from any mammal, but preferably will be from human or other primate, canine, or rodent sources. Such cells may be enriched from other cell types by affinity chromatography using publicly available procedures. Attachment of AGP-3-related protein to such cells is evaluated in the presence or absence of test compounds and the extent of binding may be determined by, for example, flow cytometry using a biotinylated antibody. Cell culture assays may be used advantageously to further evaluate compounds that score positive in protein binding assays described above.

Description of Preferred Embodiments

The following examples are offered to illustrate the invention, but should not be construed as limiting the scope thereof.

Materials and Methods

Cloning of Human AGP-3

A TNF family profile search of the Genbank dbEST data base was performed. Smith et al.(1994), Cell, 76: 959-62; Luethy et al.(1994), Protein Science, 3: 139-46. One human EST sequence (GenBank accession number T87299) was identified as a possible new member of the TNF ligand. The EST was obtained from human fetal liver spleen cDNA library (The WashU-Merck EST Project). The cDNA clone (115371 3') corresponding to the EST sequence was obtained from Genome Systems, Inc. (St. Louis, MO). The cDNA fragment was released from the pT7T3D vector with EcoRI and NotI digestion. The fragment was approximately 0.7 kb in length and was used for the subsequent full-length cloning.

The ^{32}P-dCTP-labeled T87299 cDNA fragment was used as a probe to screen a human spleen cDNA phage library (Stratagene, La Jolla, CA). Recombinant phages were plated onto E. coli strain XL1-blue at approximately 5 x 10^4 transformants per 150 mm LB plate. Nitrocellulose filters were lifted from these plates in duplicates. Filters were prehybridized in 5x SSC, 50% deionized formamide, 5x Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA for 2 hours at 42 °C. The filters were then hybridized in the same solution with the addition of 5 ng/ml of labeled probe at 42°C overnight. The filters were first washed in 2x SSC and 0.1% SDS for 10 minutes at RT twice, and then washed in 0.1x SSC and 0.1% SDS at 65 °C for 30 minutes twice. The filters were then exposed to autoradiography with intensifying screens at -80 °C overnight. Positive hybridizing plaques were determined by aligning the duplicate filters, and then picked up for subsequent

secondary or tertiary screening till single isolated positive plaque was obtained. From total of one million recombinant phage clones, 8 positive plaques were obtained.

The pBluescript phagemid was excised from phage using the ExAssist™/SOLR™ System according to the manufacturer's description (Stratagene, La Jolla, CA). The excised phagemids were plated onto freshly grown SOLR cells on LB/ampicillin plates and incubated overnight. Single bacteria colony was amplified in LB media containing 100 μg/ml ampicillin. The plasmid DNA was prepared and both strands of cDNA insert were sequenced.

The human AGP-3 cDNA (clone 13-2) is 1.1 kb in length. It encodes a LORF of 285 amino acids. FASTA search of the SwissProt database with the predicted AGP-3 protein sequence indicated that it is mostly related to human TNFα with 25% identity in C-terminal 116 amino acid overlap. Like other TNF ligand family members, human AGP-3 protein is a type II transmembrane protein, containing a short N-terminal intracellular domain (amino acids 1-46), a hydrophobic transmembrane region (amino acids 47-68) following by a long C-terminal extracellular domain (amino acid 69-285). The C-terminal extracellular domain of AGP-3 contained most of the conserved region of the TNF ligand family. Smith et al.(1994), Cell, 76: 959-62.

Cloning of Murine AGP-3

An EST sequence (Genebank accession number AA254047) encoding a potential murine AGP-3 ortholog was identified by BLAST search of Genebank dbEST database with human AGP-3 sequence. The corresponding cDNA clone (722549 5') from mouse lymph node library was obtained from Genome Systems, Inc. (St. Louis, MO). The clone contained a 0.9 kb cDNA insert which could be released by EcoRI and NotI digestion. The 0.9 kb cDNA fragment encodes an open reading frame

of 96 amino acids which shares 87% identity with the corresponding C-terminal human AGP-3 polypeptide sequence. A 0.41 kb EcoRI-XmnI fragment, which contained 290 bp coding region and 120 bp 3' non-coding region, was used as probe to screening a mouse spleen cDNA phage library (Stratagene, La Jolla, CA) for full length murine AGP-3 cDNA as described above. From one million recombinant phage clones, 6 positive plaques were obtained. The phagemid was excised from phage as described above. The plasmid DNA was prepared and both strands of cDNA insert were sequenced. The murine AGP-3 cDNA (clone S6) encodes a polypeptide of 309 amino acids. Like its human ortholog, murine AGP-3 is also a type II transmembrane region, containing a short N-terminal intracellular domain (amino acid 1-46), a hydrophobic transmembrane region (amino acid 47-68) following by a long C-terminal extracellular domain (amino acid 69-285). The human and murine AGP-3 share 68% amino acid sequence identity overall. However, the C-terminal 142 amino acid sequences share 87% identity between the two species. Preceding the highly conserved C-terminus region, there is an insertion of 30 extra amino acids in the murine AGP-3. Four out of 7 positive phage plaques were independent clones, yet they all shared the same coding sequences.

Expression of human and murine AGP-3 mRNA

Multiple human or murine tissue northern blots (Clontech, Palo Alto, CA) were probed with ^{32}P-dCTP labeled human AGP-3 0.7kb EcoRI-NotI fragment or murine AGP-3 0.41kb EcoRI-XmnI fragment, respectively. The Northern blots were prehybridized in 5x SSC, 50% deionized formamide, 5xDenhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA for 2 hours at 42 °C. The blots were then hybridized in the same solution with the addition of 5ng/ml of labeled probe at 42°C overnight. The filters were first washed in 2x SSC and 0.1%

SDS for 10 minutes at RT twice, and then washed in 0.1x SSC and 0.1% SDS at 65 °C for 30 minutes twice. The blots were then exposed to autoradiography. The human tissue northern blot analysis with human AGP-3 probe under stringent conditions revealed predominant AGP-3 transcripts with a related molecular mass of 2.4kb in peripheral blood leukocytes (Figure 4A). Weaker expression was also detected in human spleen, lung and small intestine (Figure 4A). Among murine tissues analyzed, murine AGP-3 mRNA, with a relative molecular mass of 2kb, was mainly detected in spleen, lung, liver and kidney (Figure 4B).

Overexpression of murine AGP-3 in transgenic mice

Murine AGP-3 cDNA clone S6 in pBluescript SK(-) in pBluescript was used as template to PCR the entire coding region. T3 primer

5′ AAT TAA CCC TCA CTA AAG GG 3″

SEQ ID NO: 28

was used as 5′ PCR primer. The 3′ end PCR primer, which contained a XhoI site, was

5′ TCT CCC TCG AGA TCA CGC ACT CCA GCA AGT GAG 3′

SEQ ID NO: 29

PCR reactions were carried in a volume of 50 µl with 1 unit of vent DNA polymerase (New England Biolabs) in 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.1% Triton-X100, 10 µM of each dNTP, 1 µM of each primer and 10 ng of murine AGP-3 cDNA template. Reactions were performed in 94 °C for 45 s, 55 °C for 55 S, and 72 °C for 2 minutes, for a total of 35 cycles. The PCR fragment created a XhoI site at 3′ end after the AGP-3 coding region. The 1 kb PCR fragment was purified by electrophoresis, and digested with XbaI (present in the pBluescript MCS, 80 bp upstream of AGP-3 starting Methione) and XhoI restriction enzymes. The XbaI-XhoI PCR fragment was cloned into expression vector under the control of the human β-actin promoter. Graham et al.(1997), Nature Genetics 17: 272-4;

Ray et al.(1991), Genes Dev. 5: 2265-73. The PCR fragment was sequenced to ensure no mutation. The murine AGP-3 expression plasmid was purified through two rounds of CsCl density gradient centrifugation. The purified plasmid was digested with ClaI, and a 6 kb fragment containing murine AGP-3 transgene was purified by gel electrophoresis. The purified fragment was resuspended in 5 mM Tris, pH 7.4, 0.2 mM EDTA at 2 µg/ml concentration. Single-cell embryos from BDF1 x BDF1-bred mice were injected as described (WO97 /23614). Embryos were cultured overnight in a CO_2 incubator and 15-20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

Following term pregnancy, 62 offspring were obtained from implantation of microinjected embryos. The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. Ear pieces were digested in 20 µl ear buffer (20mM Tris, pH8.0, 10mM EDTA, 0.5% SDS, 500 µg/ml proteinase K) at 55°C overnight. The sample was diluted with 200 µl of TE, and 2 µl of the ear sample was used for the PCR reaction. The 5' PCR primer

5' AAC AGG CTA TTT CTT CAT CTA CAG 3'

SEQ ID NO: 30

resided in the murine AGP-3 coding region. The 3' PCR primer

5' CTC ATC AAT GTA TCT TAT CAT GTC T 3'

SEQ ID NO: 31

resided in the vector 3' to the murine AGP-3 transgene. The PCR reactions were carried in a volume of 50 µl with 0.5 unit of Tag DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 10 µM of each dNTP, 1 µM of each primer and 2 µl of ear sample. The mixtures were first heated at 94 °C for 2 min, and the PCR reactions were performed in 94 °C for 30 s, 55 °C for 30 s, and 72 °C

for 45 s, for a total of 35 cycles. Of the 62 offspring, 10 were identified as PCR positive transgenic founders.

At 8 weeks of age, all ten transgenic founders (animal 3, 6, 9, 10, 13, 38, 40, 58, 59, and 62) and five controls (animal 7, 8, 11, 12 and 14) were sacrificed for necropsy and pathological analysis. Portions of spleen were removed, and total cellular RNA was isolated from the spleens of all the transgenic founders and negative controls using the Total RNA Extraction Kit (Qiagen Inc., Chartsworth, CA). The expression of the transgene was determined by RT-PCR. The cDNA was synthesized using the SuperScript™ Preamplification System according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD). The primer

5' CTC ATC AAT GTA TCT TAT CAT GTC T 3'

SEQ ID NO: 32

which was located in the expression vector sequence 3' to the AGP-3 transgene, was used to prime cDNA synthesis from the transgene transcripts. Ten µg total spleen RNA from transgenic founders and controls were incubated with 1 µM of primer at 70°C for 10 min, and placed on ice. The reaction was then supplemented with 10 mM Tris-HCl pH 8.3, 50 mM KCL, 2.5 mM $MgCl_2$, 10 µM of each dNTP, 0.1 mM DTT and 200 U SuperScript II RT. After incubation at 42 °C for 50 min, the reaction was stopped by heating at 72 °C for 15 min. Total RNA were digested by addition of 2 U RNase H and incubation at 37 °C for 20 min. Subsequent PCR reactions were carried out by using murine AGP-3 specific primers. The 5' PCR primer was

5' AGC CGC GGC CAC AGG AAC AG 3'

SEQ ID NO: 33

The 3' PCR primer was

5' TGG ATG ACA TGA CCC ATA G 3'

SEQ ID NO: 34

The PCR reaction was performed in a volume of 50 µl with 0.5 unit Tag DNA polymerase in 10 mM Tris-HCl pH 8.3, 50 mM KCL, 2.5mM $MgCl_2$, 10 µM of each dNTP, 1 µM of each primer and 1 µl of cDNA product. The reaction was performed at 94 °C for 30 s, 55°C for 30 S, and 72 °C for 1 min, for a total of 35 cycles. The PCR product was analyzed by electrophoresis. Transgene expression was detected in the spleen of all ten AGP-3 transgenic mice founders.

Biological activity of AGP-3

Prior to euthanasia, all animals were weighed, anesthetized by isofluorane and blood was drawn by cardiac puncture. The samples were subjected to hematology and serum chemistry analysis. The serum globulin level in all the AGP-3 transgenic mice (animal 3, 6, 9, 10, 13, 38, 40, 58, 59 and 62) increased more than 100% as compared to the control littermates (animal 7, 8, 11, 12 and 14, Table 1). Total protein level also increased correspondingly in the transgenic group, while albumin level remained the same. No significant differences in other serum chemistry or hematology parameters were observed at this age.

Radiography was performed after terminal exsanguination. There was no difference in the radiodensity or radiologic morphology of the skeleton. Upon gross dissection, major visceral organs were subject to weight analysis. The spleen weight relative to the body weight increased by approximately 45% in the AGP-3 transgenic group as compared to the control mice. The sizes of lymph nodes and Peyer's patches were also increased substantially in all the AGP-3 transgenic mice.

Following gross dissection, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The tissues collected were liver, spleen, pancreas, stomach, the entire gastrointestinal tract, kidney, reproductive organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, esophagus, thyroid, adrenals, urinary

bladder, lymph nodes and skeletal muscle. After fixation, the tissues were processed into paraffin blocks, and 3 μm sections were obtained. All sections were stained with hematoxylin and exosin, and subject to histologic analysis. The size and the number of the follicles in the spleen, lymph nodes and Peyer's patches were increased significantly in the AGP-3 transgenic mice (Figure 5, 6 and 7). The spleen, lymph node and Peyer's patches of both the transgenic and the control mice were subject to immunohistology analysis with B cell and T cell specific antibodies. The formalin fixed paraffin embedded sections were deparaffinized and hydrated to deionized water. The sections were quenched with 3% hydrogen peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat monoclonal anti-mouse B220 and CD3 (Harlan, Indianapolis, IN), respectively. The binding was detected by biotinylated rabbit anti-rat immunoglobulins and peroxidase conjugated streptavidin (BioGenex, San Ramon, CA) with DAB as chromagen (BioTek, Santa Barbara, CA). Sections were counterstained with hematoxylin. The B cell numbers, as indicated by positive B220 staining, increased significantly in the spleen, lymph nodes and Peyer's batches (Figure 5, 6, and 7). The T cell numbers, as indicated by the anti-CD3 staining, were slightly decreased. There were no differences in the morphology of the thymus between the transgenic and the control group. By immunohistology, the T cell population was similar in numbers. At 8 weeks of age, there are no distinctive morphologic changes in the liver, kidneys, or urinary, central nervous, hematopoietic, skeletal, respiratory, gastrointestinal, endocrine, or reproductive systems.

After necropsy, MLN and sections of spleen and thymus from 10 AGP-3 transgenic mice (animals 3, 6, 9, 10, 13, 38, 40, 58, 59 and 62) and 5 control littermates (animals 7, 8, 11, 12, and 14) were removed. Single cell suspensions were prepared by gently grinding the tissues with the flat end

of a syringe against the bottom of a 100 µm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells were washed twice in a 15 ml volume then counted. Approximately 1 million cells from each tissue was stained with 0.5 µg antibody in a 100 µl volume of PBS (without Calcium and Magnesium) + 0.1% Bovine Albumin + 0.01% Sodium Azide. All spleen and MLN samples were incubated with 0.5 µg CD16/32(FcγIII/II) Fc block in a 20 µl volume for 10 minutes prior to the addition of FITC or PE-conjugated monoclonal antibodies against CD90.2 (Thy-1.2), CD45R (B220), CD11b(Mac-1), Gr-1, CD4, or CD8 (PharMingen, San Diego, CA) at 2-8 °C for 30 min. The cells were washed then analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). Thymus samples were stained with FITC conjugated anti-Thy-1.2, FITC conjugated anti-CD4, and PE conjugated anti-CD8 (PharMingen, San Diego, CA).

In the MLN of the AGP-3 transgenic mice, the percentage of B220 positive B cells increased by 100% (Figure 6). The percentage of the Thy-1.2 positive T cells decreased approximately 36%, with similar reductions in both CD4(+) and CD8(+) populations. The helper CD4(+) / suppressor CD8(+) ratio remained unchanged. Similar increases in B cell and reductions in T cell populations were also observed in the spleens of the AGP-3 transgenic mice (Figure 8), though to a lesser extent. No obvious changes in staining with anti-CD11b or anti-Gr-1 antibodies were observed in the lymph node and spleen between the transgenic and the control group. In the thymus, there were no differences in the percentages of Thy-1.2(+), CD4(+), CD8(+) or CD4(+)CD8(+) populations between the AGP-3 transgenic and the control mice.

Serum Immunoglobulin and Autoantibody Analysis

Transgenic mice and control littermates were bled successively at 6, 7, 8, 9, 11, and 12 weeks of age. Serum immunoglobulin levels were

quantitated using by ELISA with Mouse Hybridoma Subtype Kit as suggested by manufacture (Boehringer Mannheim, Indianapolis, IN). Presence of autoantibodies directed against nuclear antigens and dsDNA were examined in the serum by enzyme linked immunosorbant assay (ELISA). The levels of anti-nuclear antibodies were detected using ANA screen kit (Sigma) and anti-mouse IgG peroxidase secondary antibody. Mouse serum samples were diluted 1:200 in ANA screen ELISA. For the detection of anti-dsDNA autoantibodies in serum, high binding ELISA plates were coated with plasmid DNA (Immunovision) as an antigen in the presence of methylated BSA. After blocking the non-specific sites and washing, diluted mouse serum samples were added to wells in duplicated and the binding was quantitated using horse radish peroxidase-labeled anti-mouse IgG or anti-mouse IgM reagents (Southern Biotech). A pooled positive serum from BWF1 mice and pooled negative serum from B6 mice was used as controls. Experiment for the detection of anti-histone antibodies was essentially done similar to anti-DNA ELISA except that carbonate-bicarbonate buffer (pH9.6) buffer was used as coating buffer. Serum antibody data were compared by Mann Whitney test using Sigmastat software (SPSS Science, Chicago, IL).

B Cell Survival and Proliferation Assay

Cells were isolated from spleens of 2-4 months old mice by negative selection. Briefly, B lymphocytes were purified by density gradient centrifugation and then passed over a B cells column (Accurate/ Cedarlane, Westbury, NY). Cells isolated by this method were analyzed by flow cytometry and >90% were found positive for B220 staining. Isolated B cells were cultured in MEM+10% FCS at 37°C, 5%CO_2. Cells were collected from triplicate wells daily on day 1 through day 9 and incubated with 5 µg/ml Propidium Iodide. Cells were analyzed by Flow cytometry and the percentage of dead cells was calculated. For B cell

proliferation assay, purified (10^5) B cells from B6 mice as described above were cultured in MEM+10% heat inactivated FCS in triplicate in 96 well flat bottomed plate with/without 2 μg/ml of Goat $F(ab')_2$ anti-mouse IgM (Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania) and/or indicated amount of recombinant AGP-3 for a period of 4 days at 37 °C, 5%CO_2. Proliferation was measured by an uptake of radioactive ^{3}(H) thymidine in last 18 hours of pulse. Data is shown in figure 14 as mean±standard deviation of triplicate wells.

-3; 260-3.

B Cell Hyperplasia and Hypergammaglobulinemia in AGP-3 Transgenic Mice

To gain insights into the biological function for AGP-3, transgenic mice were generated that expressed full-length murine AGP-3 protein driven by the ubiquitous 3-actin promoter. Founder mice harboring the AGP-3 transgene were identified by PCR analysis of genomic DNA samples. Transgene expression was confirmed by RT-PCR from spleen total RNA. At 8 weeks of age, ten AGP-3 transgenic mice and five control littermates were subject to necropsy and pathological analysis. The transgenic mice were of normal size and weight. However, the spleen weight relative to the body weight increased by approximately 45% in the AGP-3 transgenic group as compared to the control mice. The sizes of lymph nodes and Payer's Patches were also increased substantially in all the AGP-3 transgenic mice. Histology analysis demonstrated that the size and the number of the follicles in the spleen, lymph nodes and Payer's patches were increased significantly in the AGP-3 transgenic group (Figure 10). Immunohistology staining with B and T cell specific markers indicated the B cell numbers increased significantly in the spleen, lymph nodes and Payer's patches of the transgenic group (Figure 10). The T cell numbers, as indicated by the anti-CD3 staining, were decreased

correspondingly (Figure 10). There were no differences in the morphology and immunostaining of thymus between the transgenic and the control groups. No changes were observed in other organs or organ systems of the 8 weeks old transgenic mice, including kidney, liver, and hematopoietic tissues.

The B cell hyperplasia phenotype in the AGP-3 transgenic mice was also confirmed by flow cytometry analysis. In the mesenteric lymph nodes of the AGP-3 transgenic mice, the percentage of B220 positive B cells increased by 100% (Figure 11). The percentage of the Thy-1.2 positive T cells decreased by approximately 36%, with similar reductions in both CD4(+) and CD8(+) T cells. Similar increase in B cell and reduction in T cell populations were also observed in the spleens of the AGP-3 transgenic mice, though to a lesser extent (Figure 11). Of note, the total T cell numbers in the lymph node and spleen of AGP-3 transgenic mice were similar to the control littermates. In the thymus, there were no differences in the percentages of single positive CD4(+) or CD8(+) T cells, or CD4(+)CD8(+) populations between the AGP-3 transgenic and the control mice (Figure 11). No obvious changes in staining with anti-CD11b or anti-Gr-1 antibodies were observed in the lymph nodes and spleen between the transgenic and the control group (Figure 11). The histological and FACS analysis, together, suggested severe B cell hyperplasia phenotype in the AGP-3 transgenic mice.

We also examined B cell populations of different developmental stages by FACS analysis. No differences were observed in the percentage of the pro B (B220+IgM-), immature B (B220+IgM+), or mature B (IgM+IgD+) within spleenic B cell population of the AGP-3 transgenic mice as compared to the control littermates. In addition, the number of the spleenic CD5+ B cells in the AGP-3 transgenic mice from 1 to 9 month of age was unaltered. We also didn't detect any alteration of the CD40

expression level on B cells in the transgenic mice, suggesting that the B cell hyperplasia in the AGP-3 transgenic mice was not caused by CD40 upregulation.

In addition to the B cell hyperplasia phenotype, the AGP-3 transgenic mice also had severe hypergammaglobulinemia . The serum globulin level in AGP-3 transgenic mice increased more that 100% as compared to the control group. Total protein level also increased correspondingly in the transgenic, while albumin level remained the same. The increased B cell numbers and high serum globuiin level suggested elevated serum immunoglobulin titer. Thus we examined serum levels of IgM, IgG, IgA and IgE of AGP-3 transgenic mice from 6 to 12 weeks of age. Comparing to the same age control littermates, serum IgM, IgG, IgA and IgE were significantly increased in all age groups of AGP-3 transgenic mice. The increase found in serum IgG was not specific to any particular subclass (IgG1, IgG2a, IgG2b, and IgG3). No significant differences in other serum chemistry or hematology parameters were observed at this age. The increased serum immunoglobulin levels is likely to result directly from increased B cell number, but may also be aggravated by increased B cell antibody production.

Autoantibodies associated with lupus in AGP-3 transgenic mice

Increased humoral immunity in AGP-3 transgenic mice warranted us to look for possible phenotypes resembling B cell associated autoimmune diseases such as systemic lupus erythematosus (SLE). The common denominator in lupus patients and lupus prone mice is IgG autoantibody production, and the hallmark of this disease is the presence of elevated anti-nuclear antibodies in the serum. The emergence of anti-DNA antibodies represents one final outcome in the different murine lupus models and patients with SLE. When sera from transgenic and non-transgenic mice at various age were examined for the presence of

autoantibodies recognizing nuclear antigens or dsDNA, two different lines of AGP-3 transgenic mice began to show presence of autoantibodies at around 8 weeks of age (Table 1). The amount of anti-nuclear and anti-dsDNA antibody increased with their age in the transgenic animals (Table 1). More interestingly, at 5 and 8 months of age, AGP-3 transgenic mice showed 5-10 higher amount of anti-dsDNA antibodies compared to age matched lupus prone (NZBxNZW)F1 mice. The presence of autoantibodies in the serum of AGP-3 transgenic mice did not discriminate between gender of mice. Both IgG and IgM-antibodies to dsDNA were detected in transgenic animals. Presence of such autoantibodies was undetectable in non-transgenic littermates, as expected.

Immune Complex Deposits in the Kidney of AGP-3 Transgenic Mice

Presence of anti-DNA antibodies followed by immune complex induced renal damage is classical picture seen in lupus associated nephritis. At 5 month of age, the AGP-3 transgenic mice developed glomerular proteinaceous deposits in the kidney (Figure 13). The deposits were seen in more than 60% of the glomeruli in the transgenic mice, but absent in the control littermates. Immunohistology showed the deposits contained moderate amounts of IgG and larger amounts of IgM (Figure 13). Trichrome staining showed no deposit of connective tissues in the glomeruli at 5 month of age. There is also no evidence of any cellular proliferation or presence of inflammatory cells at this age (Figure 13). Interestingly, the kidney lesions progressed as the transgenic mice grew older. At 8 month of age, there was obvious enlargement of glomeruli in the AGP-3 transgenic mice as compared to the age matched control littermates (Figure 13G). In addition, we also detected extensive connective tissue deposits in the enlarged glomeruli (Figure 13G).

Comparing to the 5 month old mice, the 8 month old transgenic mice had increased IgG level in the glomeruli immune complex deposits (Figure 13I). Majority of the glomeruli in the AGP-3 transgenic mice were affected. We also performed serum and urine chemistry analysis of 5 month old and 8 month old AGP-3 transgenic along with the control littermates. No significant differences were noticed in the 5 month old AGP-3 transgenic mice. However, in the 8 month old mice, we observed increases in serum blood urea nitrogen (BUN) and calcium levels and decrease in serum phosphate level. In addition, the 8 month old AGP-3 mice also had increased protein level in the urine. These changes, together, suggest the onset of renal failure in the 8 month old AGP-3 transgenic mice. In conclusion, the high serum autoantibodies followed by the kidney lesions in theAGP-3 transgenic mice clearly resemble to the pathological progression in the SLE patients and lupus prone mice.

AGP-3 Stimulates B Cell Survival and Proliferation : a Possible Mechanism for Autoimmunity

The B cell hyperplasia phenotype in the AGP-3 transgenic mice might arise from increased B cell survival and/or increased B cell proliferation. We first compared the viability of B cells from AGP-3 transgenic mice with that of the control littermates. B cells were isolated from both transgenic or control mice and incubated in minimal essential medium supplemented with 10% heat inactivated fetal bovine serum. Viability of the B cells was measured by FACS analysis for Propidium Iodide uptake (Figure 14A). By day 3, 30% of B cells isolated from the control mice were dead, whereas only 10% of B cells from AGP-3 transgenic mice were dead. By day 5, 70% of B cells from AGP-3 mice were still viable, whereas only 15% of B cell from control littermates were viable. By day 9, almost 50% of the AGP-3 transgenic B cells still remained viable. Therefore, transgenic expression of AGP-3 prolonged B cell

viability. It remains to be determined if this B cell survival stimuli result directly from AGP-3 action on B cells or through its modulation of the immune system.

Recently Schneider et al (Schneider et al., 1999, and Moore et al., 1999) reported co-stimulation of B cell proliferation by BAFF/BLYS with anti-IgM. We found that AGP-3 alone can also stimulates B cell proliferation in a dose dependent manner with an ED_{50} of approximately 3ng/ml (Figure 14B, upper). A ten fold increase of B cell proliferation was detected by AGP-3 treatment at 10 ng/ml concentration as compared to the untreated cells. In our experiment, anti-IgM alone at 2 μg/ml concentration increased B cell proliferation by 24 fold. Treatment with anti-IgM (2 μg/ml) in combination with various doses of AGP-3 led to dose dependent increase of B cell proliferation, with a maximal 13 fold increase as compared anti-IgM treatment alone and a total of 320 fold increase as compared to the untreated cells. Thus, AGP-3 is a potent B cell stimulatory factor. The increased B cell survival and proliferation may together contribute to the B cell hyperplasia and autoimmune lupus like changes in the AGP-3 transgenic mice.

Table 1: Lupus associated autoantibodies in the serum of AGP-3 transgenic mice.

Autoantibodies	Age (months)	AGP-3 tg (n)	Non-tg littermates (n)	p value
Antinuclear antibodies (IgG)[a]	2-3	7^ (9)	1*(8)	
	5-6	9 (9)	1*(8)	
	8-9	8 (8)	1*(6)	
Anti-dsDNA (IgG)[b]	<2	697±284 (7)	277±67 (7)	NS
	3-4	842±351 (7)	235±49 (7)	<.005
	6-7	2515±428 (5)	970±344 (7)	<.019
	8-10	12293±6767 (11)	1070±602 (12)	<.017
Anti-dsDNA (IgM)[b]	<2	275±33 (7)	46±5 (7)	<.001
	3-4	1684±920 (7)	63±13 (7)	<.003
	6-7	6998±5515 (5)	98±14 (7)	<.001
	8-10	13712±9147 (11)	79±14 (12)	<.001
Anti-Histone (Ig) [b]	<2	741±264 (7)	52±8 (7)	<.001
	3-4	837±436 (7)	53±14 (7)	<.003
	6-7	4220±933 (5)	60±10 (7)	<.001
	8-10	16555±4618 (11)	295±173 (12)	<.001

^ includes two weak positive.

* Weak positive

a: Data is shown as number of ANA positive (mean+2sd of transgene negative littermates) mice using ANA screen kit.

b: Data is represented as mean±SE for each group. Values are shown as Units/ml.

NS: not significant

Bacterial Expression of AGP-3 protein

PCR amplification employing the primer pairs and templates described below are used to generate various forms of human AGP3 proteins. One primer of each pair introduces a TAA stop codon and a unique XhoI or SacII site following the carboxy terminus of the gene. The other primer of each pair introduces a unique NdeI site, a N-terminal methionine, and optimized codons for the amino terminal portion of the gene. PCR and thermocycling is performed using standard recombinant DNA methodology. The PCR products are purified, restriction digested, and inserted into the unique NdeI and XhoI or SacII sites of vector pAMG21 (ATCC accession no. 98113) and transformed into the prototrophic E. coli 393 or 2596. Other commonly used E. coli expression vectors and host cells are also suitable for expression. After transformation, the clones are selected, plasmid DNA is isolated and the sequence of the AGP3 binding protein insert is confirmed.

pAMG21-Human AGP3 protein [128-285]

This construct was engineered to be C-terminal 158 amino acids of human AGP3 and have the following N-terminal and C-terminal residues:

NH_2-MNSRNKR ——GALKLL-COOH.

SEQ ID NO: 35

The template to be used for PCR was human AGP3 in pCDNA3.1(+) vector. Oligonucleotides #1761-31 and #1761-33 were the primer pair to be used for PCR and cloning this gene construct.

1761-31:
5'-ATT TGA TTC TAG AAG GAG GAA TAA CAT ATG AAC AGC CGT AAT AAG CGT GCC GTT CAG GGT -3'
(SEQ ID NO:36)

1761-33:

5'-CCG CGG ATC CTC GAG TTA CAG CAG TTT CAA TGC ACC AAA AAA TGT -3'

(SEQ ID NO:37)

pAMG21-Human FLAG-AGP3 protein [128-285]

This construct was engineered to be C-terminal 158 amino acids of human AGP3 preceded with FLAG epitope. The construct encoded following following N-terminal and C-terminal residues:

NH_2-MDYKDDDDKKLNSRNKR------GALKLL-COOH

(SEQ ID NO: 38)

The template to be used for PCR was human AGP3 in pCDNA3.1(+) vector. Oligonucleotides #1761-32 and #1761-33 were the primer pair to be used for PCR and cloning this gene construct.

1761-32:

5'-GAC GAT GAC AAG AAG CTT AAC AGC CGT AAT AAG CGT GCC GTT CAG GGT -3'

(SEQ ID NO:39)

1761-33:

5'-CCG CGG ATC CTC GAG TTA CAG CAG TTT CAA TGC ACC AAA AAA TGT -3'

(SEQ ID NO:37)

E. coli were induced during fermentation, the lysate was applied to Q Sepharose FF (Pharmacia, Piscaataway, NJ) equilibrated in 10 mM Mes pH 6.0 and eluted with 50- 400 mM NaCl gradient over 30 column volumes. Fractions containing AGP-3 were pooled and loaded onto a Q Sepharose HP column (Pharmacia, Piscataway, NJ) equilibrated in 10 mM Tris-HCL pH 8.5. AGP-3 was eluted with an increasing linear NaCl gradient (50 mM-200 mM) over 30 column volumes. Endotoxin was removed by application to Sp HiTRAP column (Pharmacia, Piscataway, NJ) pH 4.8 and eluted with 100-500 mM NaCl in 10 mM sodium acetate pH 4.8 over 25 column volumes. Final endotoxin level of the purified

protein is approximately 0.2 EU/mg. The purified human AGP-3 is truncated at residue Arg133 as indicated by N-terminal sequencing and has a molecular weight of 16.5 KDa by reducing SDS-PAGE. The purified human FLAG-AGP-3 protein is confirmed by N-terminal sequence analysis of the protein. The FLAG-AGP3 protein is recognized by M2 monoclonal antibody against FLAG epitope (Kodak, New Haven, CT).

For europium labeling of the protein, human AGP-3 (lot# 092299) was dialyzed into 50 mM sodium carbonate pH 9. Europium labeling reagent (Wallac Delfia reagent lot# 704394) was dissolved in the same buffer. AGP-3 protein was mixed with a 20-fold molar excess of labeling reagent for 24 hours at room temperature. The mixture was then placed on a Sephadex G-25 column which had been equilibrated in 50 mM Tris-HCl pH 7.8, 150 mM NaCl. The protein was eluted from the column with the same buffer. Protein concentration was determined using the BCA method (Pierce Chemical Co.).

Abbreviations

Abbreviations as used throughout this specification are defined as follows, unless otherwise defined in specific instances.

CDR	complementarity determining region
dsDNA	double-stranded DNA
EST	expressed sequence tag
ORF	open reading frame
SDS	sodium dodecyl sulfate
TNF	tumor necrosis factor

* * *

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the

appended claims cover all such equivalent variations that come within the scope of the invention as claimed.

What is claimed is:

1. An isolated or recombinant polypeptide having a sequence comprising SEQ ID NO: 25, wherein said polypeptide does not comprise SEQ ID NOS: 2, 4, or 5 or a sequence with 90% identity thereto.
2. The polypeptide of Claim 1, further comprising SEQ ID NO: 26.
3. The polypeptide of Claim 1 having a sequence comprising SEQ ID NO: 27.
4. The polypeptide of Claim 1, wherein said polypeptide does not comprise SEQ ID NOS: 2, 4, or 5 or a sequence with 80% identity to SEQ ID NOS: 2, 4, or 5.
5. The polypeptide of Claim 1, comprising an Fc-region.
6. The polypeptide of Claim 1, wherein the polypeptide has the structure

$$(X^1)_a\text{-}F^1\text{-}(X^2)_b$$

wherein:

F^1 is a vehicle;

X^1 and X^2 are each independently selected from $\text{-}(L^1)_c\text{-}P^1$, $\text{-}(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2$, $\text{-}(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3$, and $\text{-}(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3\text{-}(L^4)_f\text{-}P^4$

P^1, P^2, P^3, and P^4 are each independently selected from SEQ ID NOS: 6, 25, 26, and 27;

L^1, L^2, L^3, and L^4 are each independently linkers; and

a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1.

7. The composition of matter of Claim 6 of the formula

$$X^1\text{-}F^1$$

or

$$F^1\text{-}X^2.$$

8. The composition of matter of Claim 6 of the formula

$$F^1\text{-}(L^1)_c\text{-}P^1.$$

9. The composition of matter of Claim 6 of the formula

$$F^1\text{-}(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2.$$

10. The composition of matter of Claim 6 wherein F^1 is an IgG Fc domain.

11. The composition of matter of Claim 6 wherein F^1 is an IgG1 Fc domain.

12. The polypeptide of Claim 1, wherein the polypeptide comprises an antibody sequence in which one or more amino acids from antibody CDR regions are replaced by sequences selected from SEQ ID NOS: 6, 25, 26, and 27.

13. The polypeptide of Claim 12, wherein a first CDR region is replaced by SEQ ID NO: 25 and a second CDR region is replaced by SEQ ID NO: 26.

14. The polypeptide of Claim 1, wherein the polypeptide comprises a sequence selected from SEQ ID NOS: 7 TO 24 and 40 shown in Figure 9, except that the B/B' region is replaced by SEQ ID NO: 25.

15. The polypeptide of Claim 1, wherein the polypeptide comprises a sequence selected from SEQ ID NOS: 7 TO 24 and 40 shown in Figure 9, except that the B/B' region is replaced by SEQ ID NO: 25 and the E/F region is replaced by SEQ ID NO: 26.

16. The polypeptide of Claim 1, wherein the polypeptide comprises a sequence selected from SEQ ID NOS: 7 TO 24 and 40 shown in Figure 9, except that the B/I region is replaced by SEQ ID NO: 27.

17. A polypeptide of Claim 1 capable of eliciting B cell growth, survival, or activation in mesenteric lymph nodes.

18. The protein of any of Claims 1, 2, 3, 4, 14, 15, 16, or 17, wherein the protein is covalently linked to a water-soluble polymer or a carbohydrate.

19. The protein of Claim 18, wherein the polymer is polyethylene glycol.

20. The protein of Claim 18, wherein the carbohydrate is dextran.

21. An isolated nucleic acid encoding a protein of any of Claims 1 to 17.

22. The nucleic acid of Claim 21 including one or more codons preferred for Escherichia coli expression.

23. The nucleic acid of Claim 21 having a detectable label attached thereto.

24. An expression vector comprising the nucleic acid of Claim 21.

25. A host cell transformed or transfected with the expression vector of Claim 24.

26. The host cell of Claim 25, wherein the cell is a prokaryotic cell.

27. The host cell of Claim 26, wherein the cell is Escherichia coli.

28. A method to assess the ability of a candidate compound to bind to an AGP-3 related protein comprising:

(a) incubating a polypeptide of Claim 1 with the candidate compound under conditions that allow binding; and

(b) measuring the bound compound.

29. A method of regulating expression of an AGP-3 related protein in an animal comprising administering to the animal a nucleic acid complementary to the nucleic acid of Claim 22.

30. A pharmaceutical composition comprising a therapeutically effective amount of a protein of Claim 1 in a pharmaceutically acceptable carrier, adjuvant, solubilizer, stabilizer and/or anti-oxidant.

31. A method of modulating B cell growth, survival, or activation in a mammal, which comprises administering a therapeutically effective amount of a modulator of an AGP-3 related protein.

32. The method of Claim 31, wherein the B cell growth, survival, or activation takes place in mesenteric lymph nodes.

33. An antibody that specifically binds to SEQ ID NOS: 25, 26, or 27.

34. The antibody of claim 33, wherein the antibody is a monoclonal antibody.

35. The antibody of claim 33, wherein the antibody was generated by phage display.

36. A method of modulating B cell growth, survival, or activation in a mammal comprising administering a therapeutically effective amount of the antibody of Claim 33.

37. The method of Claim 36, wherein the B cell growth, survival, or activation takes place in mesenteric lymph nodes.

38. A method to modulate hybridoma cell antibody production, which comprises treating hybridoma cells with the compound of Claim 1.

FIG. 1A

```
        10                  30                  50
GAATTCGGCACGAGCTGAGGGGTGAGCCAAGCCCTGCCATGTAGTGCACGCAGGACATCA
        70                  90                  110
ACAAACACAGATAACAGGAAATGATCCATTCCCTGTGGTCACTTATTCTAAAGGCCCCAA
      130                 150                 170
CCTTCAAAGTTCAAGTAGTGATATGGATGACTCCACAGAAAGGGAGCAGTCACGCCTTAC
                      M   D   D   S   T   E   R   E   Q   S   R   L   T
      190                 210                 230
TTCTTGCCTTAAGAAAAGAGAAGAAATGAAACTGAAGGAGTGTGTTTCCATCCTCCCACG
 S   C   L   K   K   R   E   E   M   K   L   K   E   C   V   S   I   L   P   R
      250                 270                 290
GAAGGAAAGCCCCTCTGTCCGATCCTCCAAAGACGGAAAGCTGCTGGCTGCAACCTTGCT
 K   E   S   P   S   V   R   S   S   K   D   G   K   L   L   A   A   T   L   L
      310                 330                 350
GCTGGCACTGCTGTCTTGCTGCCTCACGGTGGTGTCTTTCTACCAGGTGGCCGCCCTGCA
 L   A   L   L   S   C   C   L   T   V   V   S   F   Y   Q   V   A   A   L   Q
      370                 390                 410
AGGGGACCTGGCCAGCCTCCGGGCAGAGCTGCAGGGCCACCACGCGGAGAAGCTGCCAGC
 G   D   L   A   S   L   R   A   E   L   Q   G   H   H   A   E   K   L   P   A
      430                 450                 470
AGGAGCAGGAGCCCCCAAGGCCGGCCTGGAGGAAGCTCCAGCTGTCACCGCGGGACTGAA
 G   A   G   A   P   K   A   G   L   E   E   A   P   A   V   T   A   G   L   K
      490                 510                 530
AATCTTTGAACCACCAGCTCCAGGAGAAGGCAACTCCAGTCAGAACAGCAGAAATAAGCG
 I   F   E   P   P   A   P   G   E   G   N   S   S   Q   N   S   R   N   K   R
      550                 570                 590
TGCCGTTCAGGGTCCAGAAGAAACAGTCACTCAAGACTGCTTGCAACTGATTGCAGACAG
 A   V   Q   G   P   E   E   T   V   T   Q   D   C   L   Q   L   I   A   D   S
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FIG. 1B

```
      610                 630                 650
TGAAACACCAACTATACAAAAAGGATCTTACACATTTGTTCCATGGCTTCTCAGCTTTAA
 E  T  P  T  I  Q  K  G  S  Y  T  F  V  P  W  L  L  S  F  K
      670                 690                 710
AAGGGGAAGTGCCCTAGAAGAAAAAGAGAATAAAATATTGGTCAAAGAAACTGGTTACTT
 R  G  S  A  L  E  E  K  E  N  K  I  L  V  K  E  T  G  Y  F
      730                 750                 770
TTTTATATATGGTCAGGTTTTATATACTGATAAGACCTACGCCATGGGACATCTAATTCA
 F  I  Y  G  Q  V  L  Y  T  D  K  T  Y  A  M  G  H  L  I  Q
      790                 810                 830
GAGGAAGAAGGTCCATGTCTTTGGGGATGAATTGAGTCTGGTGACTTTGTTTCGATGTAT
 R  K  K  V  H  V  F  G  D  E  L  S  L  V  T  L  F  R  C  I
      850                 870                 890
TCAAAATATGCCTGAAACACTACCCAATAATTCCTGCTATTCAGCTGGCATTGCAAAACT
 Q  N  M  P  E  T  L  P  N  N  S  C  Y  S  A  G  I  A  K  L
      910                 930                 950
GGAAGAAGGAGATGAACTCCAACTTGCAATACCAAGAGAAAATGCACAAATATCACTGGA
 E  E  G  D  E  L  Q  L  A  I  P  R  E  N  A  Q  I  S  L  D
      970                 990                1010
TGGAGATGTCACATTTTTTGGTGCATTGAAACTGCTGTGACCTACTTACACCATGTCTGT
 G  D  V  T  F  F  G  A  L  K  L  L
     1030                1050                1070
AGCTATTTTCCTCCCTTTCTCTGTACCTCTAAGAAGAAAGAATCTAACTGAAAATACCAA
     1090                1110                1130
AAAAAAAAAAAAAAAAAAAAAAAAAGTAGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAA
     1150                1170
AAAAAAAAAAAAAAAAAAAAAACTCGGAGGGGG
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FIG. 2A

```
        10                  30                  50
GAATTCGGCACGAGCTCCAAAGGCCTAGACCTTCAAAGTGCTCCTCGTGGAATGGATGAG
                                                  M  D  E
        70                  90                  110
TCTGCAAAGACCCTGCCACCACCGTGCCTCTGTTTTTGCTCCGAGAAAGGAGAAGATATG
S  A  K  T  L  P  P  P  C  L  C  F  C  S  E  K  G  E  D  M
      130                 150                 170
AAAGTGGGATATGATCCCATCACTCCGCAGAAGGAGGAGGGTGCCTGGTTTGGGATCTGC
K  V  G  Y  D  P  I  T  P  Q  K  E  E  G  A  W  F  G  I  C
      190                 210                 230
AGGGATGGAAGGCTGCTGGCTGCTACCCTCCTGCTGGCCCTGTTGTCCAGCAGTTTCACA
R  D  G  R  L  L  A  A  T  L  L  L  A  L  L  S  S  S  F  T
      250                 270                 290
GCGATGTCCTTGTACCAGTTGGCTGCCTTGCAAGCAGACCTGATGAACCTGCGCATGGAG
A  M  S  L  Y  Q  L  A  A  L  Q  A  D  L  M  N  L  R  M  E
      310                 330                 350
CTGCAGAGCTACCGAGGTTCAGCAACACCAGCCGCCGCGGGTGCTCCAGAGTTGACCGCT
L  Q  S  Y  R  G  S  A  T  P  A  A  A  G  A  P  E  L  T  A
      370                 390                 410
GGAGTCAAACTCCTGACACCGGCAGCTCCTCGACCCCACAACTCCAGCCGCGGCCACAGG
G  V  K  L  L  T  P  A  A  P  R  P  H  N  S  S  R  G  H  R
      430                 450                 470
AACAGACGCGCTTTCCAGGGACCAGAGGAAACAGAACAAGATGTAGACCTCTCAGCTCCT
N  R  R  A  F  Q  G  P  E  E  T  E  Q  D  V  D  L  S  A  P
      490                 510                 530
CCTGCACCATGCCTGCCTGGATGCCGCCATTCTCAACATGATGATAATGGAATGAACCTC
P  A  P  C  L  P  G  C  R  H  S  Q  H  D  D  N  G  M  N  L
      550                 570                 590
AGAAACATCATTCAAGACTGTCTGCAGCTGATTGCAGACAGCGACACGCCGACTATACGA
R  N  I  I  Q  D  C  L  Q  L  I  A  D  S  D  T  P  T  I  R
```

FIG. 2B

```
      610                 630                 650
AAAGGAACTTACACATTTGTTCCATGGCTTCTCAGCTTTAAAAGAGGAAATGCCTTGGAG
K  G  T  Y  T  F  V  P  W  L  L  S  F  K  R  G  N  A  L  E
      670                 690                 710
GAGAAAGAGAACAAAATAGTGGTGAGGCAAACAGGCTATTTCTTCATCTACAGCCAGGTT
E  K  E  N  K  I  V  V  R  Q  T  G  Y  F  F  I  Y  S  Q  V
      730                 750                 770
CTATACACGGACCCCATCTTTGCTATGGGTCATGTCATCCAGAGGAAGAAAGTACACGTC
L  Y  T  D  P  I  F  A  M  G  H  V  I  Q  R  K  K  V  H  V
      790                 810                 830
TTTGGGGACGAGCTGAGCCTGGTGACCCTGTTCCGATGTATTCAGAATATGCCCAAAACA
F  G  D  E  L  S  L  V  T  L  F  R  C  I  Q  N  M  P  K  T
      850                 870                 890
CTGCCCAACAATTCCTGCTACTCGGCTGGCATCGCGAGGCTGGAAGAAGGAGATGAGATT
L  P  N  N  S  C  Y  S  A  G  I  A  R  L  E  E  G  D  E  I
      910                 930                 950
CAGCTTGCAATTCCTCGGGAGAATGCACAGATTTCACGCAACGGAGACGACACCTTCTTT
Q  L  A  I  P  R  E  N  A  Q  I  S  R  N  G  D  D  T  F  F
      970                 990                1010
GGTGCCCTAAAACTGCTGTAACTCACTTGCTGGAGTGCGTGATCCCCTTCCCTCGTCTTC
G  A  L  K  L  L
     1030                1050                1070
TCTGTACCTCCGAGGGAGAAACAGACGACTGGAAAAACTAAAAGATGGGGAAAGCCGTCA
     1090                1110                1130
GCGAAAGTTTTCTCGTGACCCGTTGAATCTGATCCAAACCAGGAAATATAACAGACAGCC
     1150                1170                1190
```

FIG. 3A

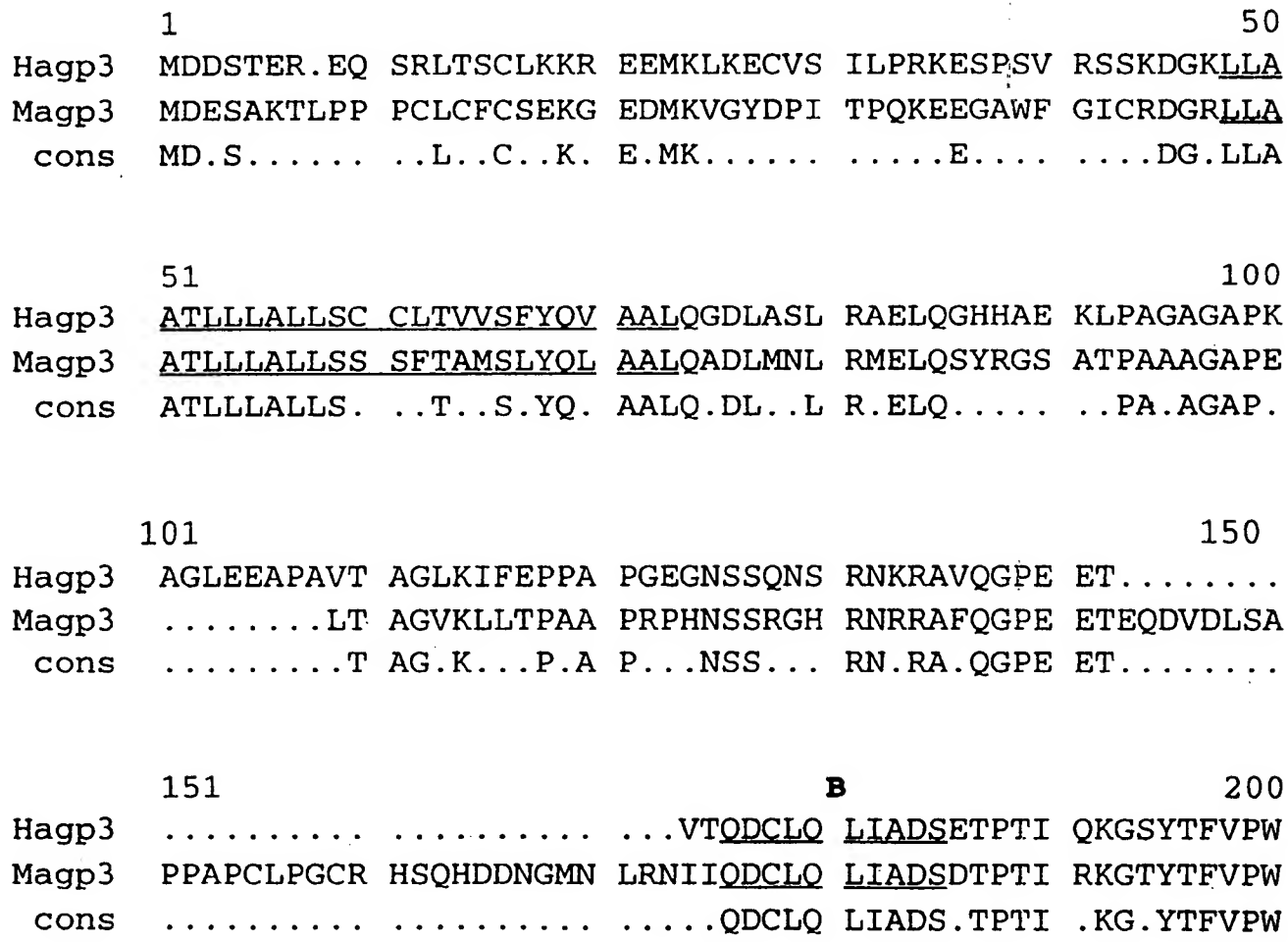


FIG. 3B

```
          B'        C'        C              D                  E          250
Hagp3   LLSFKRGSAL EEKENKILVK ETGYFFIYGQ VLYTDKTYAM GHLIQRKKVH
Magp3   LLSFKRGNAL EEKENKIVVR QTGYFFIYSQ VLYTDPIFAM GHVIQRKKVH
 cons   LLSFKRG.AL EEKENKI.V. .TGYFFIY.Q VLYTD...AM GH.IQRKKVH

          251     F                        G                H          300
Hagp3   VFGDELSLVT LFRCIQNMPE TLPNNSCYSA GIAKLEEGDE LQLAIPRENA
Magp3   VFGDELSLVT LFRCIQNMPK TLPNNSCYSA GIARLEEGDE IQLAIPRENA
 cons   VFGDELSLVT LFRCIQNMP. TLPNNSCYSA GIA.LEEGDE .QLAIPRENA

          301          I     317
Hagp3   QISLDGDVTF FGALKLL
Magp3   QISRNGDDTF FGALKLL
 cons   QIS..GD.TF FGALKLL
```

FIG. 4A

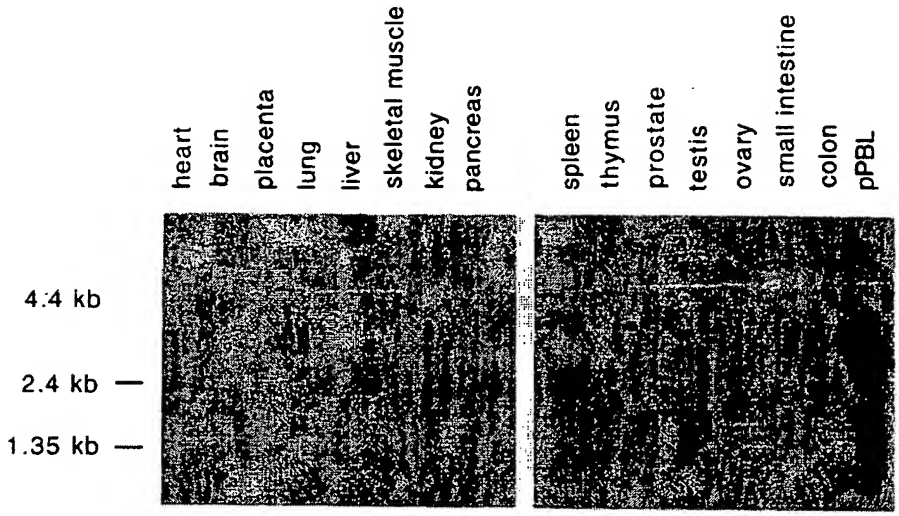

heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
spleen
thymus
prostate
testis
ovary
small intestine
colon
pPBL
4.4 kb
2.4 kb
1.35 kb

FIG. 4B

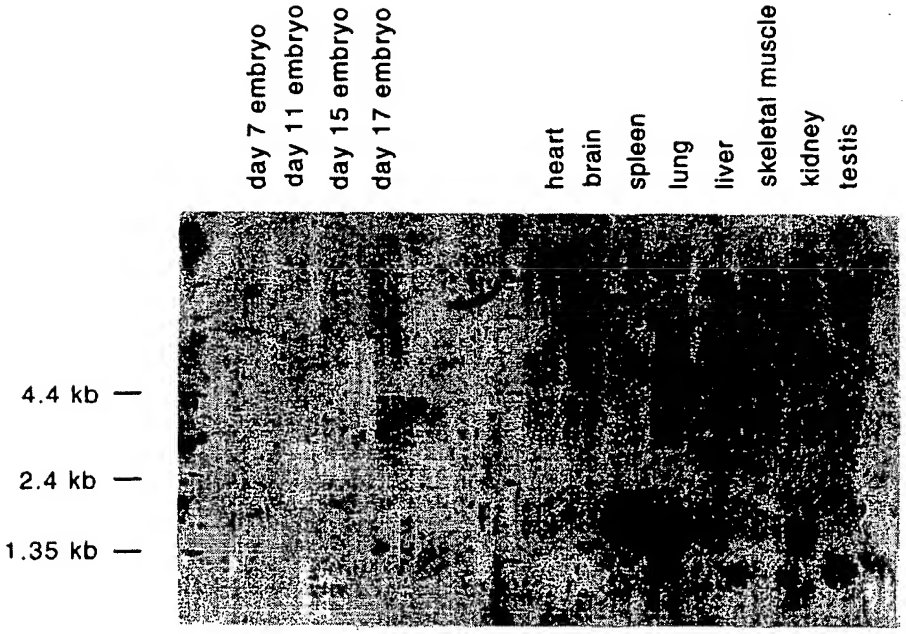

day 7 embryo
day 11 embryo
day 15 embryo
day 17 embryo
heart
brain
spleen
lung
liver
skeletal muscle
kidney
testis
4.4 kb
2.4 kb
1.35 kb

FIG. 5A

FIG. 5B

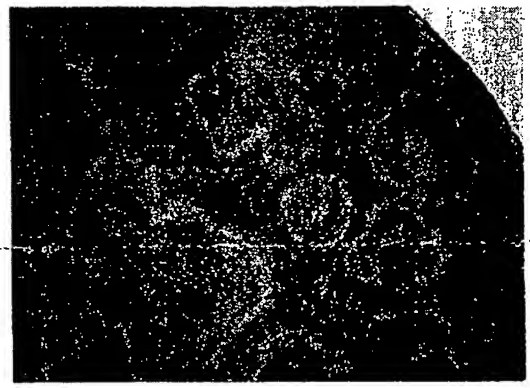


FIG. 5C

FIG. 5D

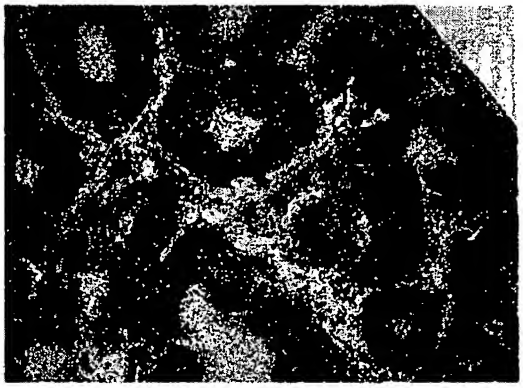


FIG. 5E

FIG. 5F

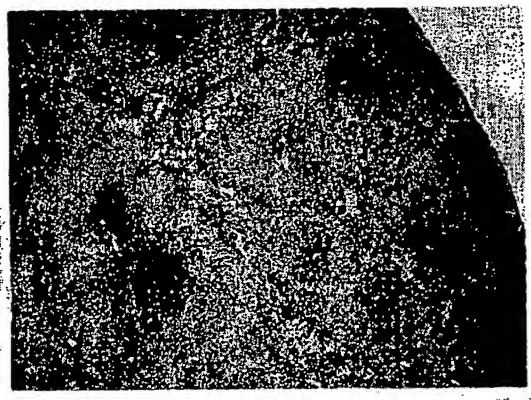


FIG. 6A

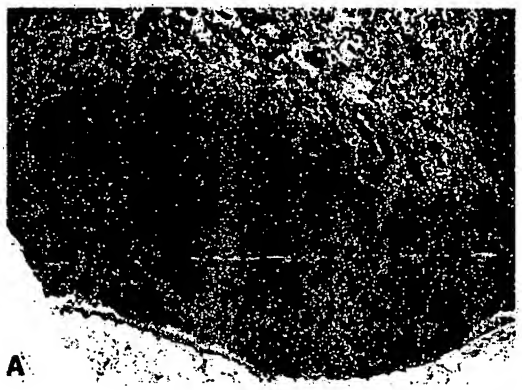


FIG. 6B

FIG. 6C

FIG. 6D

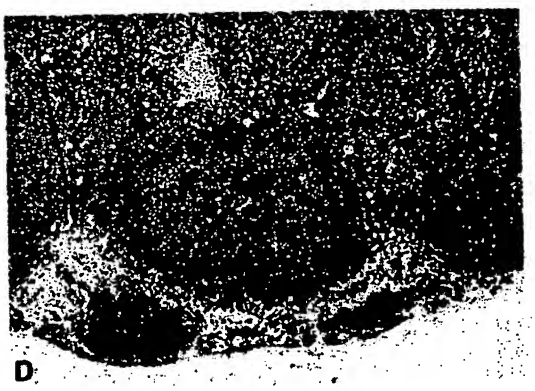


FIG. 6E

FIG. 6F

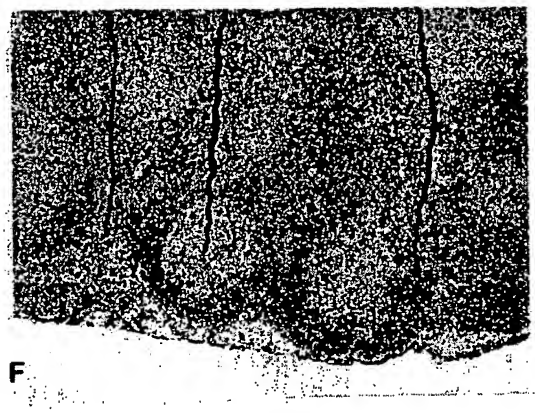


FIG. 7A

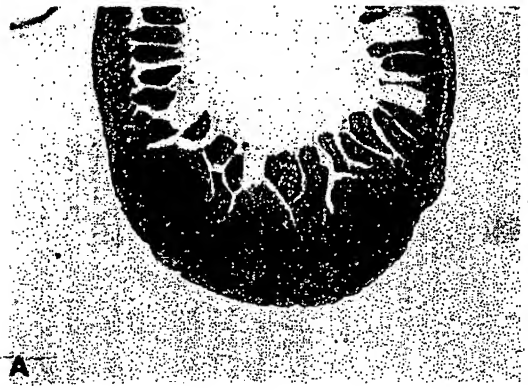


FIG. 7B

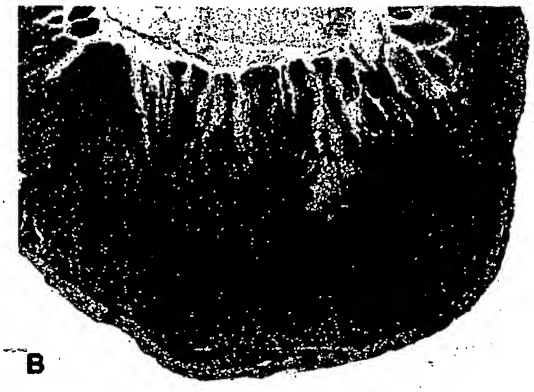


FIG. 7C

FIG. 7D

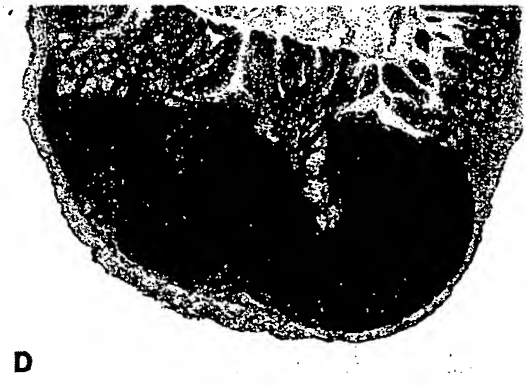


FIG. 7E

FIG. 7F

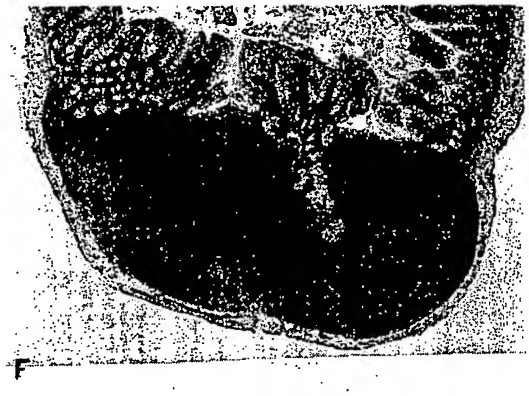


FIG. 8A

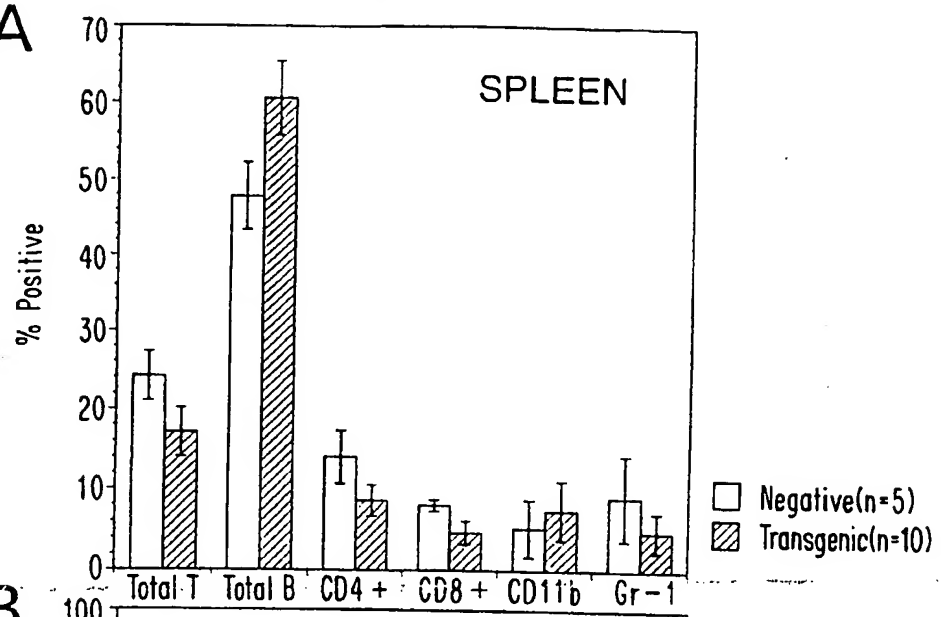


FIG. 8B

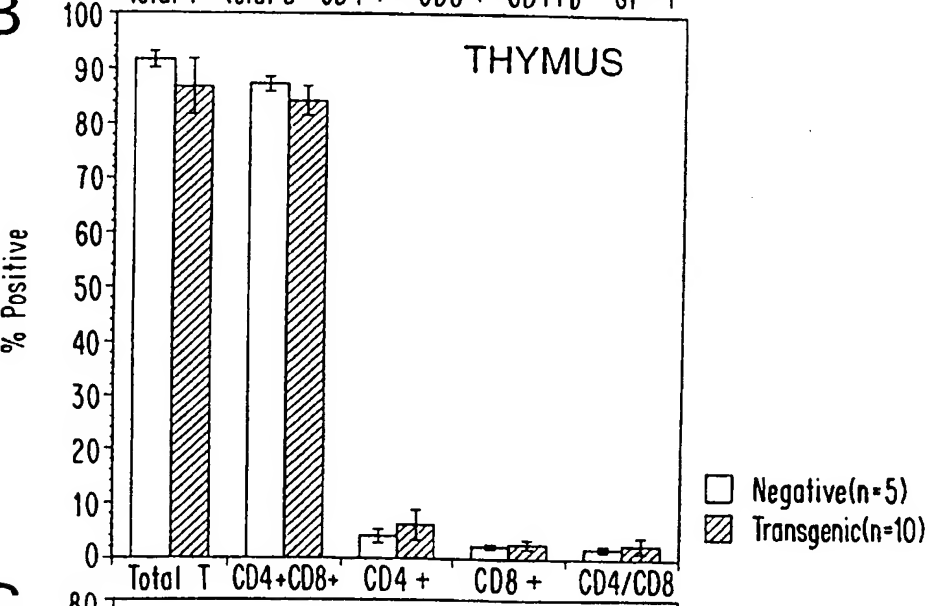


FIG. 8C

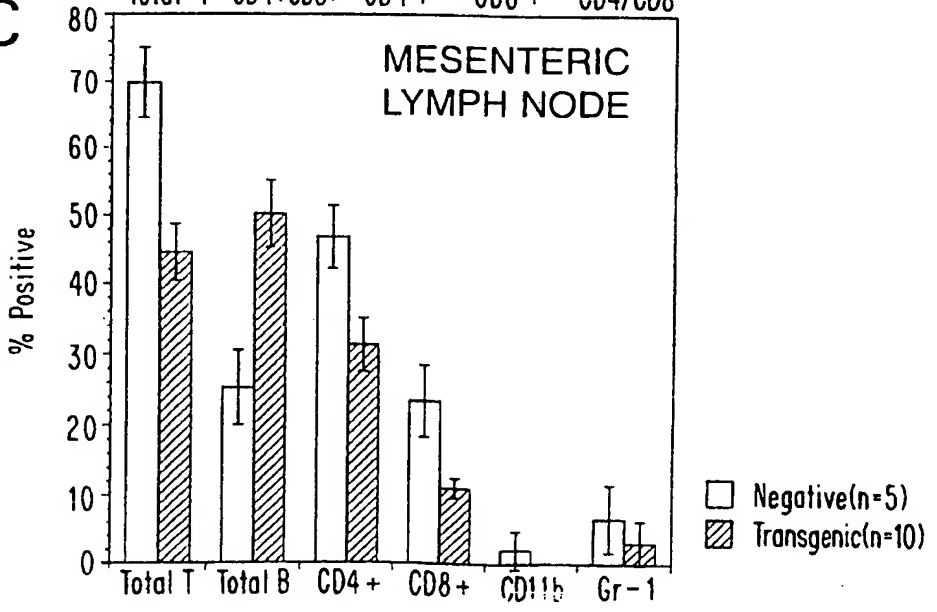
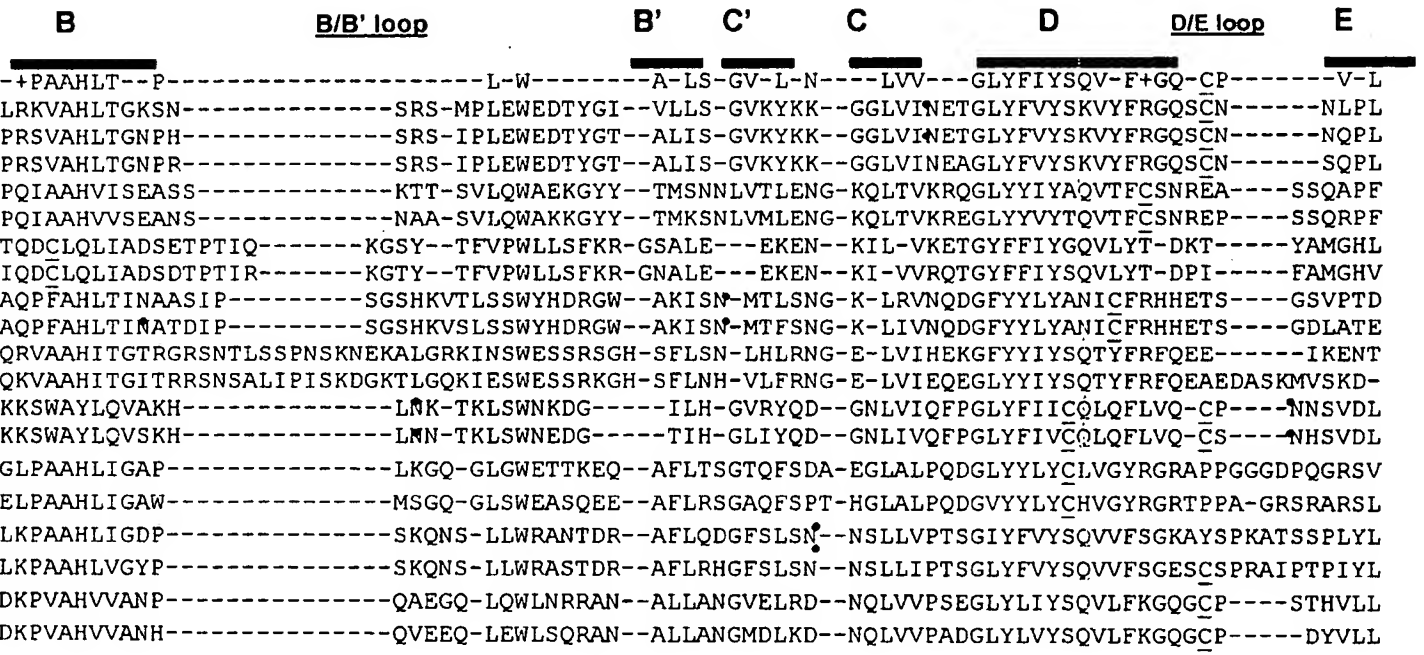
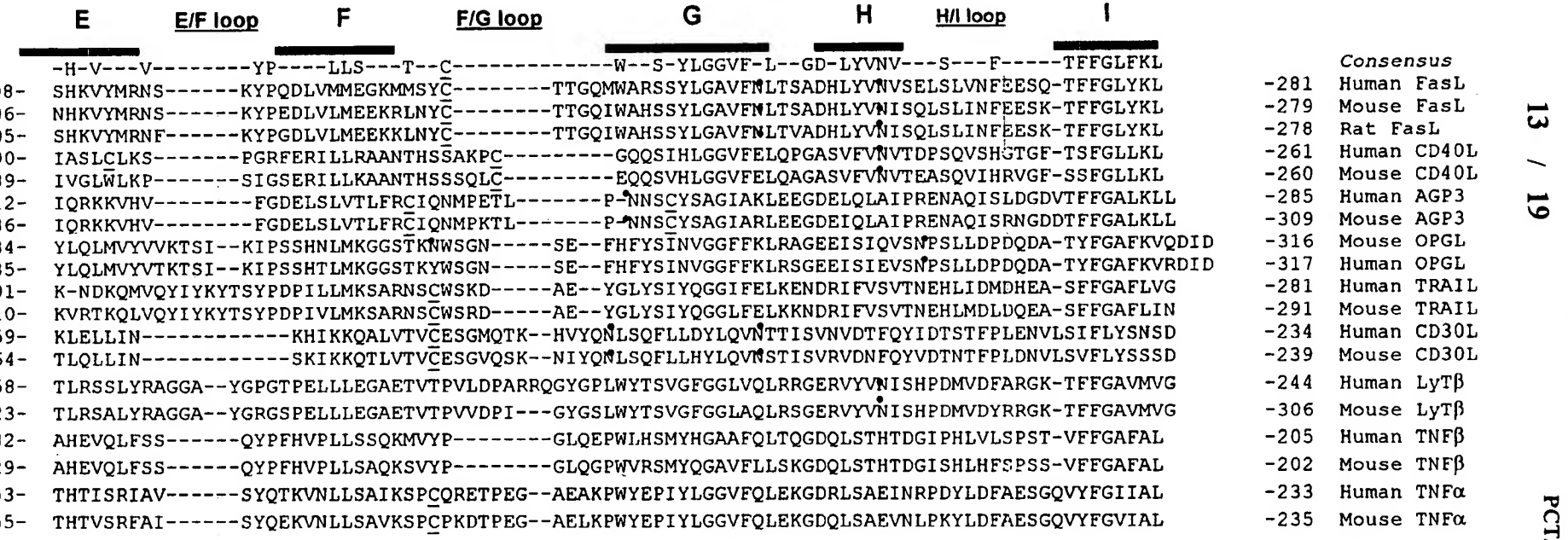


FIG. 9A



SUBSTITUTE SHEET (RULE 26)

FIG. 9B



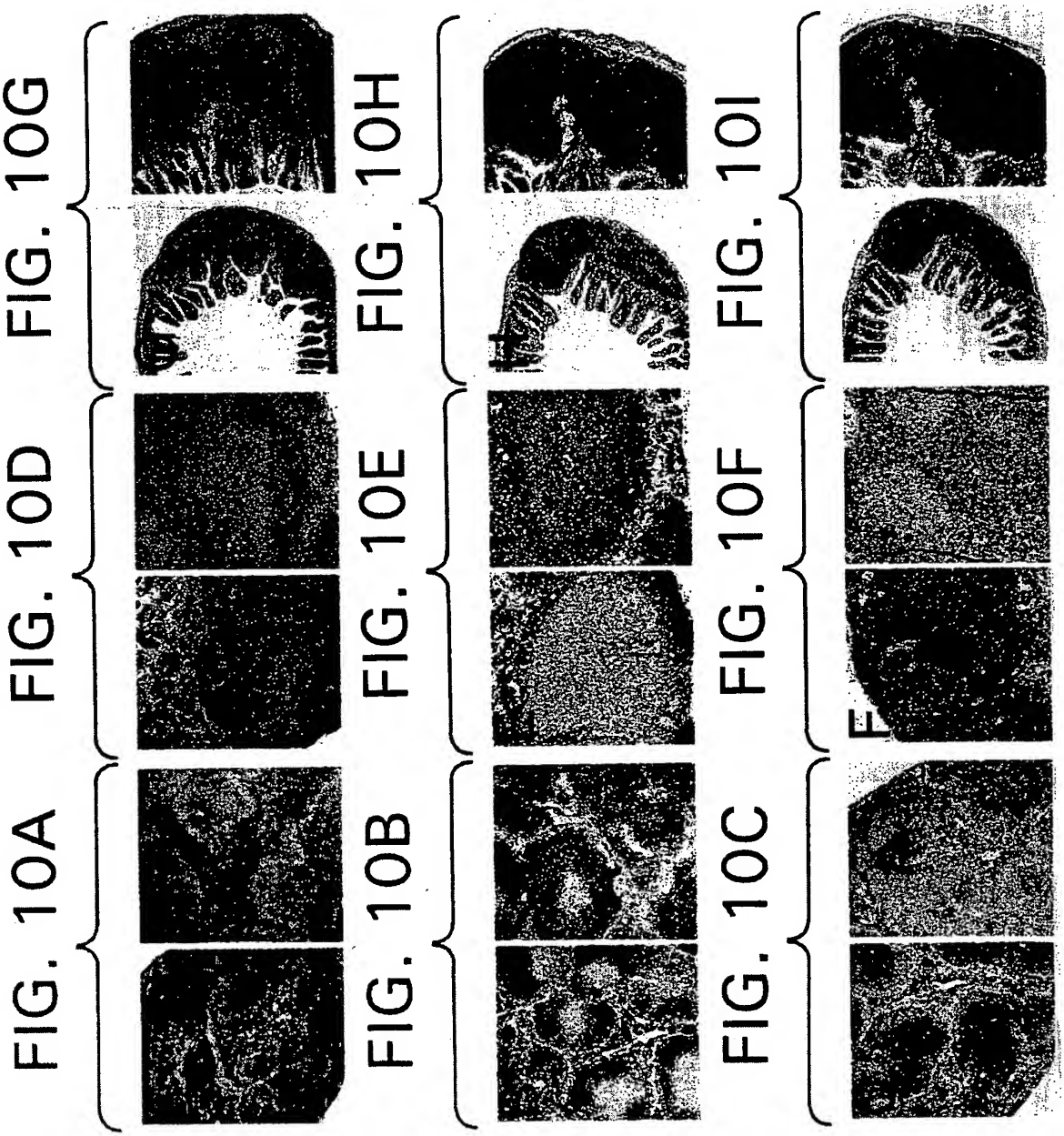


FIG. 10A
FIG. 10B
FIG. 10C
FIG. 10D
FIG. 10E
FIG. 10F
FIG. 10G
FIG. 10H
FIG. 10I

FIG. 11A

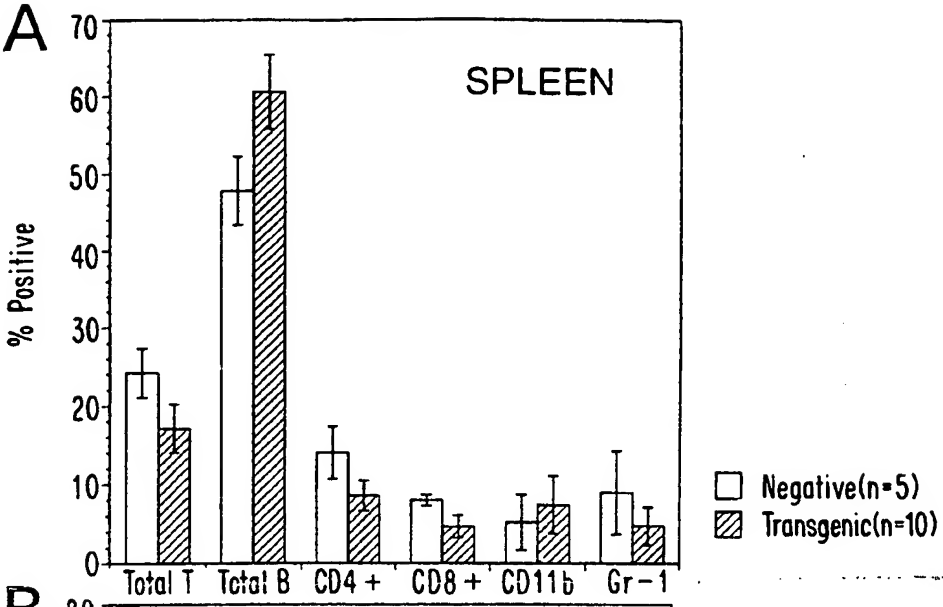


FIG. 11B

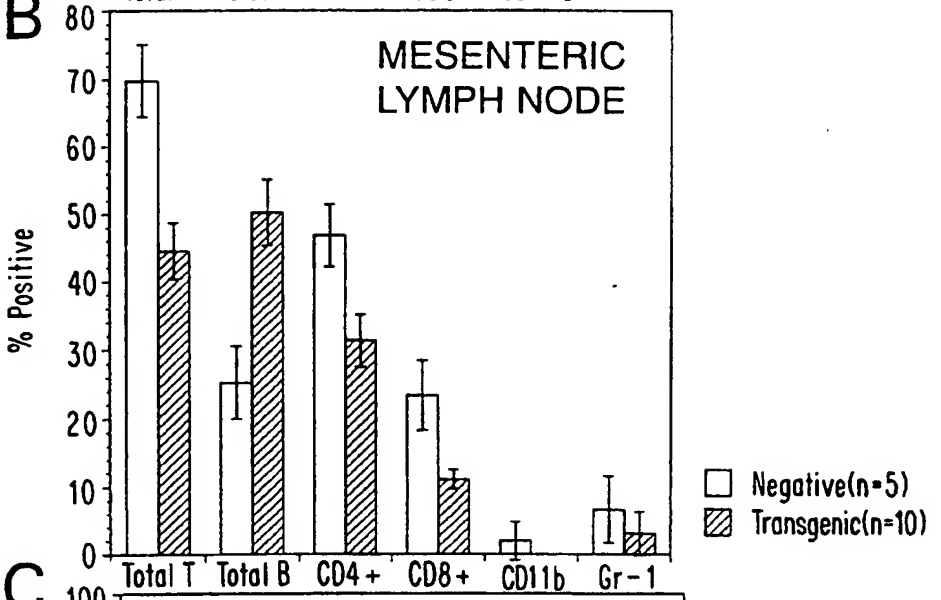


FIG. 11C

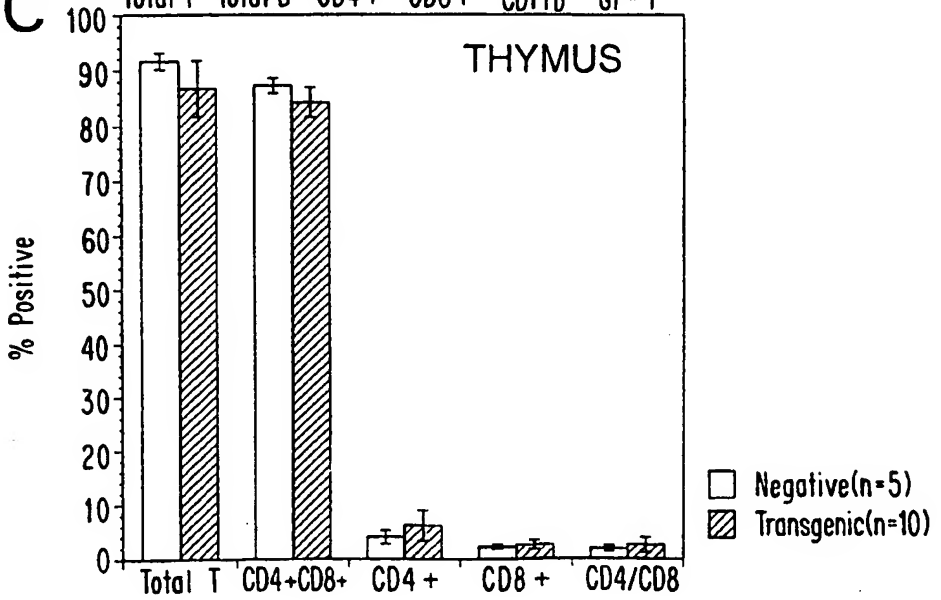


FIG. 12A

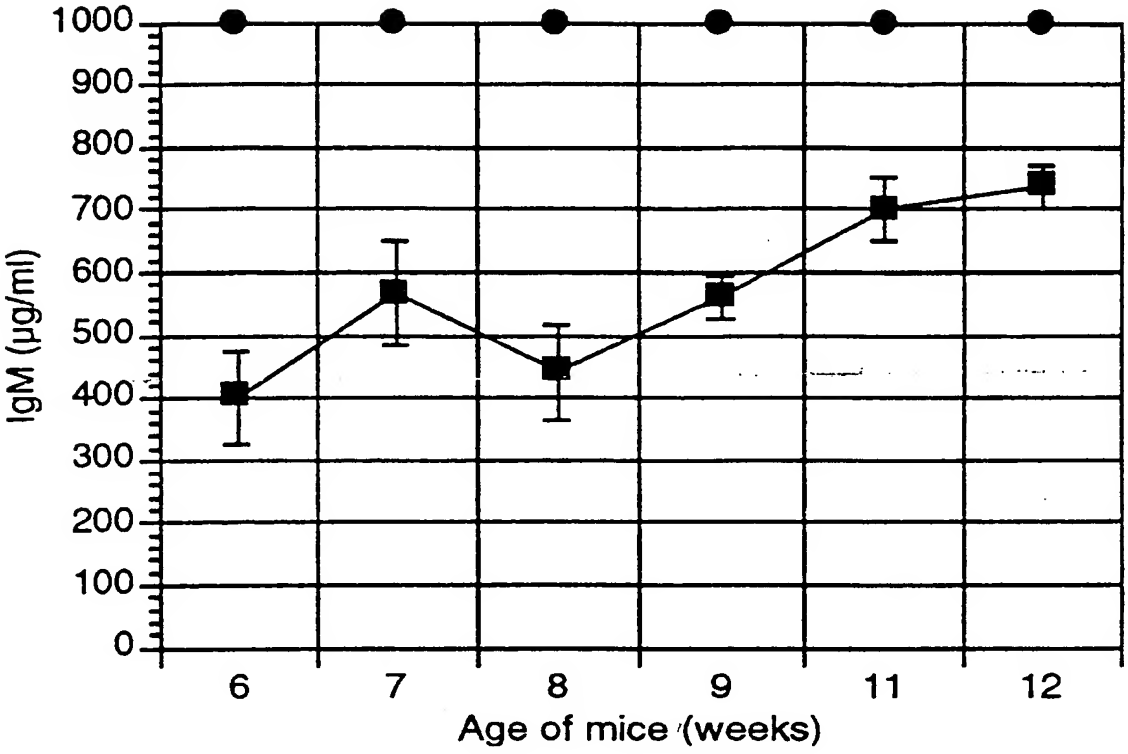


FIG. 12B

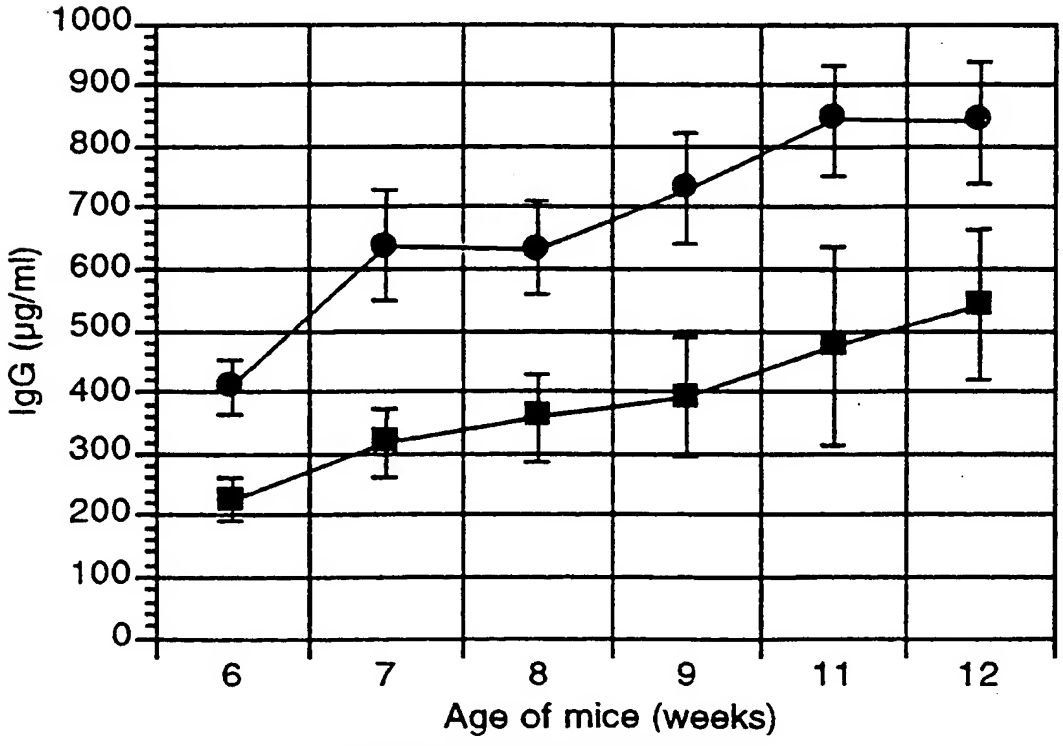


FIG. 12C

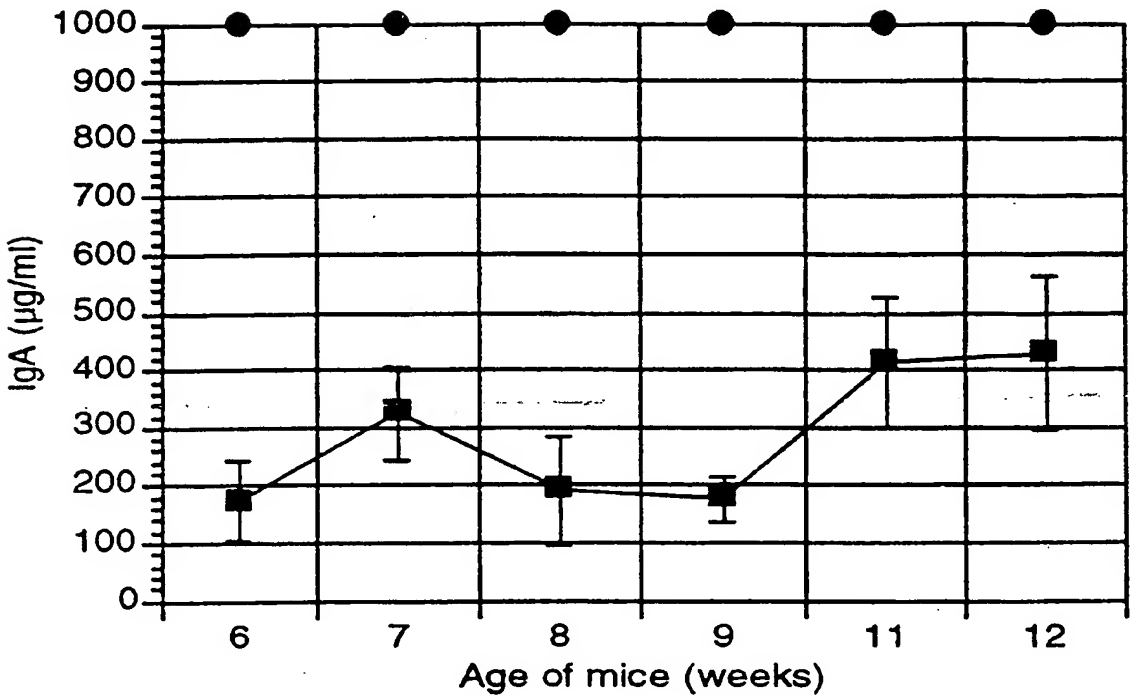
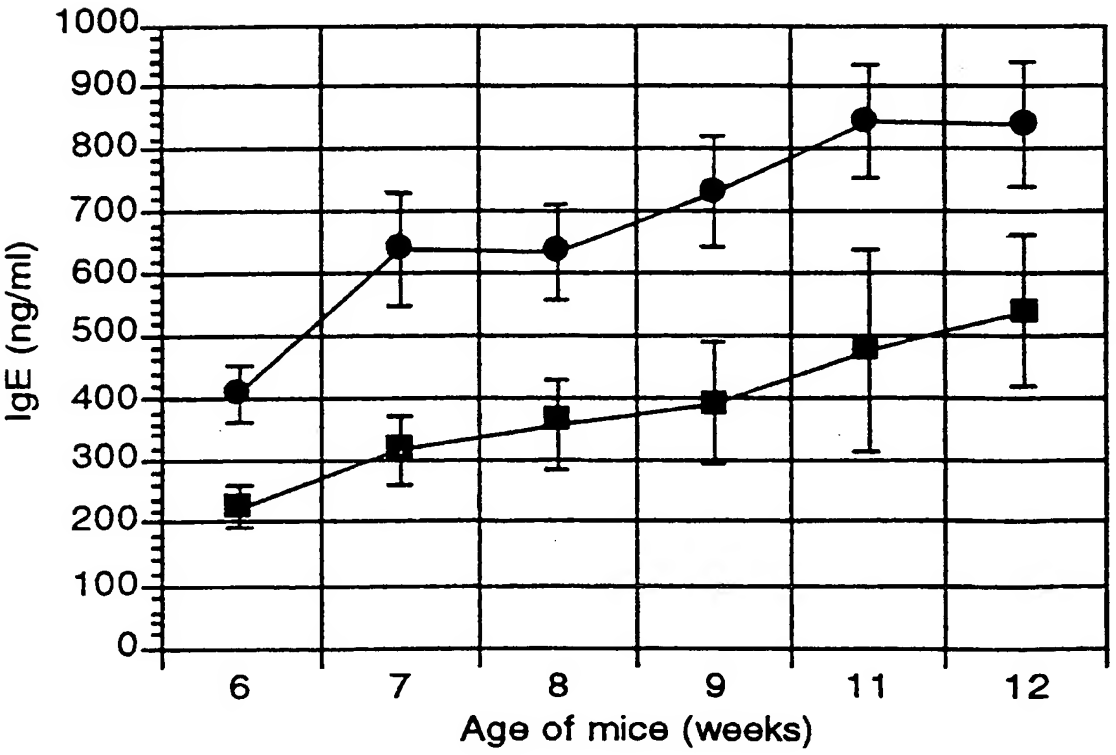


FIG. 12D



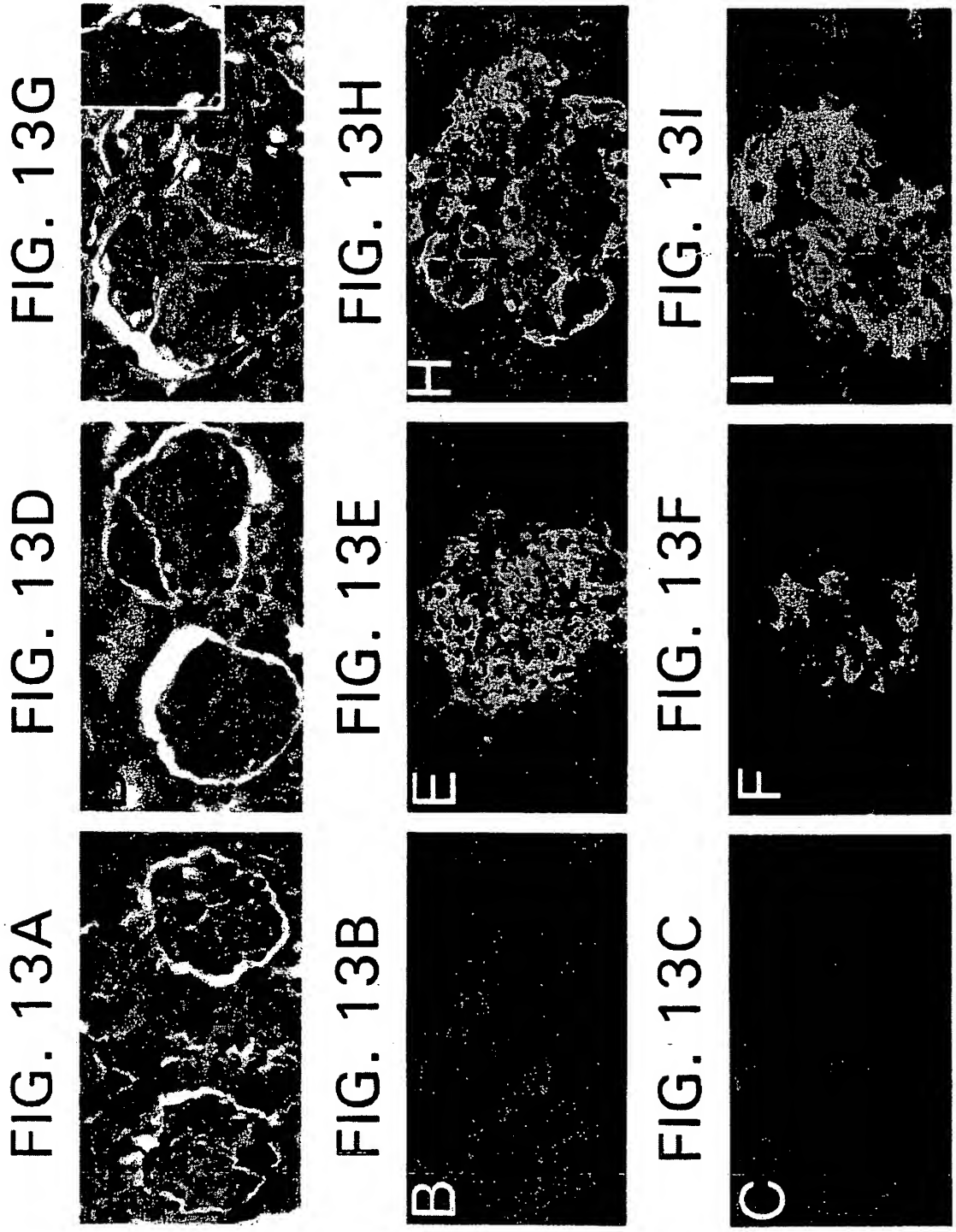


FIG. 13A
FIG. 13B
FIG. 13C
FIG. 13D
FIG. 13E
FIG. 13F
FIG. 13G
FIG. 13H
FIG. 13I
B
C
E
F
H
I

FIG. 14A

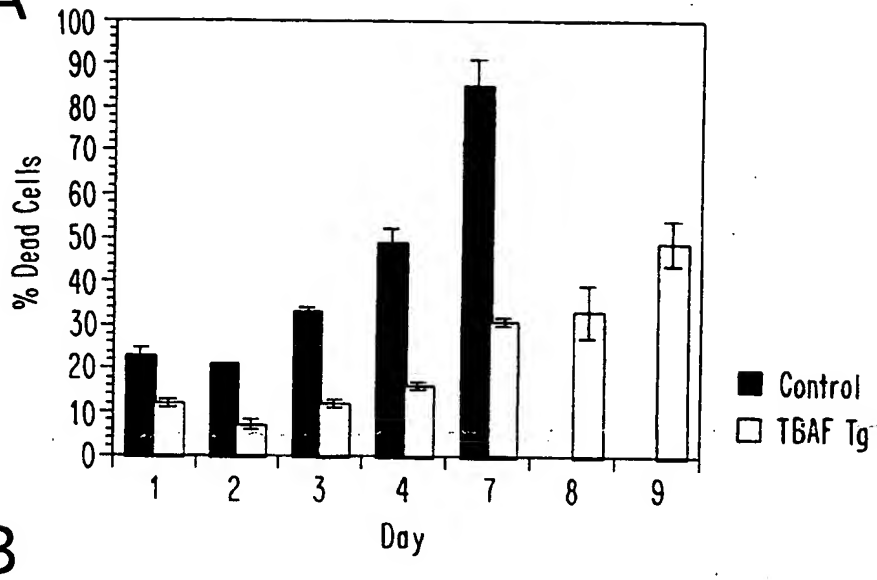


FIG. 14B

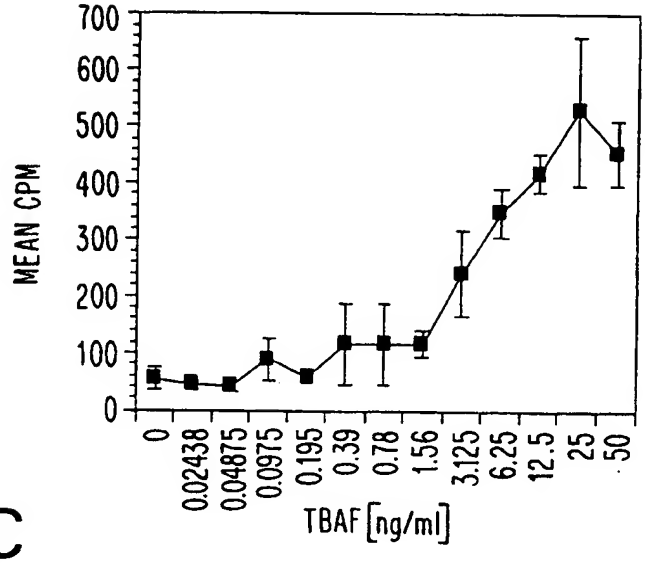
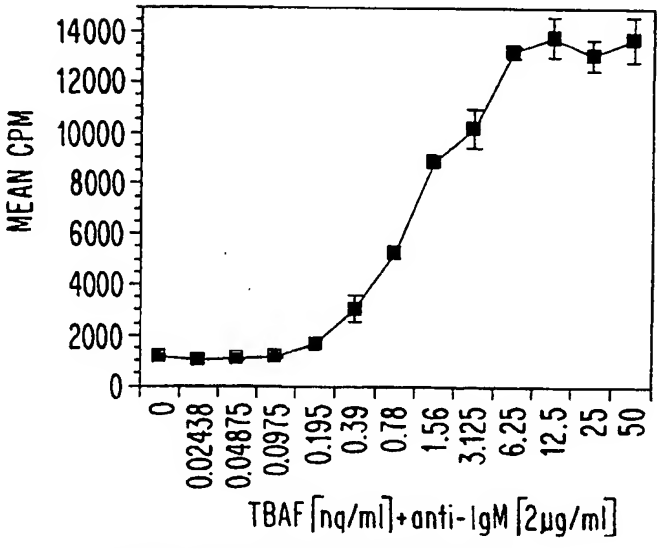


FIG. 14C



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/03653

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/705 C12N15/62 C07K19/00 C12N15/70
A61K48/00 A61K38/19 C07K16/22 C07K16/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 46686 A (AMGEN INC) 11 December 1997 (1997-12-11) page 3, line 25 -page 16, line 15	1,5-9, 18-21, 23-25, 28-33, 36,37
Y	WO 98 49305 A (AMGEN INC ;BOYLE WILLIAM J (US); WOODEN SCOTT (US)) 5 November 1998 (1998-11-05) page 4, line 8 -page 8, line 35; example 1	1,5-9, 18-21, 23-25, 28-33, 36,37
X	WO 97 33617 A (PROTEIN DESIGN LABS INC ;QUEEN CARY L (US); SCHNEIDER WILLIAM P (U) 18 September 1997 (1997-09-18) the whole document	1,3,6-9, 14-16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 September 2000	02/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/03653

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 18307 A (SANDOZ AG ;BUEHLER THOMAS (CH); SANDOZ LTD (CH); SANDOZ AG (DE)) 22 May 1997 (1997-05-22) page 1, last paragraph -page 2, paragraph 3 page 3, paragraphs 3,4 page 7, paragraph 3 -page 9, last paragraph	1,3, 14-16,18
X	EP 0 869 180 A (SMITHKLINE BEECHAM CORP) 7 October 1998 (1998-10-07) SEQ.ID.N.2 and 4 the whole document	1,2, 14-16
A	WO 98 55621 A (MASIAKOWSKI PIOTR ;REGENERON PHARMA (US); VALENZUELA DAVID (US)) 10 December 1998 (1998-12-10) SEQ.ID.N.4 and 6. the whole document	1,3, 14-16
A	EP 0 675 200 A (MOCHIDA PHARM CO LTD ;OSAKA BIOSCIENCE INST (JP)) 4 October 1995 (1995-10-04) SEQ.ID.N.3	3,14-16
A	YAMAGUCHI K ET AL: "Characterisation of structural domains of human osteoclastogenesis inhibitory factor" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 9, no. 273, 27 February 1998 (1998-02-27), pages 5117-5123, XP002077021 ISSN: 0021-9258 the whole document	1,14-16
A	DANILENKO D M (REPRINT) ET AL: "AGP - 1, a novel member of the tumor necrosis factor family, induces hepatic necrosis and inflammation in transgenic mice" FASEB JOURNAL,US,FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, BETHESDA, MD, vol. 11, no. 3, 28 February 1997 (1997-02-28), XP002045026 ISSN: 0892-6638 abstract	1
P,X	WO 99 26977 A (TSCHOPP JURG ;BIOGEN INC (US)) 3 June 1999 (1999-06-03) the whole document	1,5-9,30

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/03653

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	SCHNEIDER P ET AL: "BAFF, A NOVEL LIGAND OF THE TUMOR NECROSIS FACTOR FAMILY, STIMULATES B CELL GROWTH" JOURNAL OF EXPERIMENTAL MEDICINE,TOKYO,JP, vol. 189, no. 11, 7 June 1999 (1999-06-07), pages 1747-1756, XP000915409 ISSN: 0022-1007 the whole document	14-17
E	WO 00 24782 A (AMGEN INC) 4 May 2000 (2000-05-04) the whole document	1,6-13

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 6-9

Present claims 6-9 relate to an extremely large number of possible compounds. In fact, the claims contain so many options, that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely immunoglobulin Fc-regions as those mentioned in the description at pages 18-20.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/03653

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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(54) Title: TNF-RELATED PROTEINS

(57) Abstract: A member of the tumor necrosis factor family and associated antibodies and uses are described. This member is primarily expressed in B cells and its expression correlates to increases in the number of B cells and immunoglobulins produced. The human ortholog contains 285 amino acids; the mouse ortholog, 309 amino acids. The protein is a type II transmembrane protein and has an amino terminal cytoplasmic domain, a transmembrane domain, and a carboxy terminal extracellular domain. TNF-related proteins of the invention may be membrane-associated or in soluble form, recombinantly produced or isolated after natural production. The invention provides for nucleic acids encoding such TNF-related proteins, vectors and host cells expressing the polypeptides, and methods for producting recombinant porteins. Antibodies, fragments, and related fusion proteins and derivatives may be used as agonists or antagonists of AGP-3 related activity.

TNF-RELATED PROTEINS

Cross-reference to Related Applications

This specification is related to U.S. provisional application nos. 60/119,906, filed February 12, 1999 and 60/166,271, filed November 18, 1999, respectively, both of which are hereby incorporated by reference in their entirety.

Field of the Invention

The present invention relates to proteins that are involved in inflammation and immunomodulation, particularly in B cell growth, survival, or activation. The invention further relates to proteins related to the tumor necrosis factor (TNF)/nerve growth factor (NGF) superfamily and related nucleic acids, expression vectors, host cells, and binding assays. The specification also describes compositions and methods for the treatment of immune-related and inflammatory, autoimmune and other immune-related diseases or disorders, such as rheumatoid arthritis (RA), Crohn's disease (CD), lupus, and graft versus host disease (GvHD).

The invention also relates to methods and compositions for the treatment of inflammatory and immune-related diseases and disorders using the receptors.

Background of the Invention

After years of study in necrosis of tumors, tumor necrosis factors (TNFs) α and β were finally cloned in 1984. The ensuing years witnessed the emergence of a superfamily of TNF cytokines, including fas ligand (FasL), CD27 ligand (CD27L), CD30 ligand (CD30L), CD40 ligand (CD40L), TNF-related apoptosis-inducing ligand (TRAIL, also designated AGP-1), osteoprotegerin binding protein (OPG-BP or OPG ligand), 4-1BB ligand, LIGHT, APRIL, and TALL-1. Smith et al. (1994), Cell **76**: 959-962; Lacey et al. (1998), Cell **93**: 165-176; Chichepotiche et al. (1997), J. Biol.

Chem. 272: 32401-32410; Mauri et al. (1998), Immunity 8: 21-30; Hahne et al. (1998), J.Exp. Med. 188: 1185-90; Shu et al. (1999), J. Leukocyte Biology 65: 680-3. This family is unified by its structure, particularly at the C-terminus. In addition, most members known to date are expressed in immune compartments, although some members are also expressed in other tissues or organs, as well. Smith et al. (1994), Cell 76: 959-62. All ligand members, with the exception of LT-α, are type II transmembrane proteins, characterized by a conserved 150 amino acid region within the C-terminal extracellular domain. Though restricted to only 20-25% identity, the conserved 150 amino acid domain folds into a characteristic β-pleated sheet sandwich and trimerizes. This conserved region can be proteolyticaly released, thus generating a soluble functional form. Banner et al. (1993), Cell 73: 431-445.

Many members within this ligand family are expressed in lymphoid enriched tissues and play important roles in immune system development and modulation. Smith et al. (1994). For example, TNFα is mainly synthesized by macrophages and is an important mediator for inflammatory responses and immune defenses. Tracey & Cerami (1994), Annu. Rev. Med. 45: 491-503. Fas-L, predominantly expressed in activated T cell, modulates TCR-mediated apoptosis of thymocyts. Nagata, S. & Suda, T. (1995) Immunology Today 16: 39-43; Castrim et al. (1996), Immunity 5: 617-27. CD40L, also expressed by activated T cells, provides an essential signal for B cell survival, proliferation and immunoglobulin isotype switching. Noelle (1996), Immunity 4: 415-9.

The cognate receptors for most of the TNF ligand family members have been identified. These receptors share characteristic multiple cysteine-rich repeats within their extracellular domains, and do not possess catalytic motifs within cytoplasmic regions. Smith et al. (1994). The receptors signal through direct interactions with death domain proteins (e.g. TRADD, FADD, and RIP) or with the TRAF proteins (e.g.

TRAF2, TRAF3, TRAF5, and TRAF6), triggering divergent and overlapping signaling pathways, e.g. apoptosis, NF-κB activation, or JNK activation. Wallach et al. (1999), Annual Review of Immunology 17: 331-67. These signaling events lead to cell death, proliferation, activation or differentiation. The expression profile of each receptor member varies. For example, TNFR1 is expressed on a broad spectrum of tissues and cells; whereas the cell surface receptor of OPGL is mainly restricted to the osteoclasts. Hsu et al. (1999) Proc. Natl. Acad. Sci. USA 96: 3540-5. It is therefore an object of the invention to identify proteins and nucleic acids related to TNFs. Such proteins are believed to play a role in inflammatory and immune processes, suggesting their usefulness in treating autoimmune and inflammatory disorders.

Summary of the Invention

In accordance with the present invention, the inventors describe a novel member of the tumor necrosis factor family. The novel TNF ligand family member is herein called AGP-3. Unlike other members of the family, the receptor for AGP-3 is primarily expressed in B cells, and its expression correlates to increases in the number of B cells and immunoglobulins produced.

The natural, preferred human ortholog is here called hAGP-3 and contains 285 amino acids; the mouse ortholog (mAGP-3), contains 309 amino acids. The AGP-3 protein is a type II transmembrane protein and has an amino terminal cytoplasmic domain, a transmembrane domain, and a carboxy terminal extracellular domain. TNF-related proteins of the invention may be membrane-associated or in soluble form, recombinantly produced or isolated after natural production. The present specification demonstrates that AGP-3 is a potent B cell stimulatory factor. Interestingly, the AGP-3 transgenic mice also developed autoantibodies

and kidney immune complex deposits, a phenotype resembling lupus patients and lupus prone mice.

The invention provides for nucleic acids encoding AGP-3, vectors and host cells expressing the polypeptides, and methods for producing recombinant proteins. Antibodies or fragments thereof that specifically bind AGP-3 are also provided.

The subject proteins may be used in assays to identify cells and tissues that express AGP-3 or proteins related to AGP-3 and to identify new AGP-3-related proteins. Methods of identifying compounds that interact with AGP-3 proteins are also provided. Such compounds include nucleic acids, peptides, proteins, carbohydrates, lipids or small molecular weight organic molecules and may act either as agonists or antagonists of AGP-3 or AGP-3 R-protein activity.

AGP-3-related proteins are involved in B cell growth, survival, and activation, particularly in lymph node, spleen, and Peyer's patches. AGP-3 agonists and antagonists (e.g., antibodies to AGP-3) thus modulate B cell response and may be used to treat diseases characterized by inflammatory processes or deregulated immune response, such as RA, GvHD, CD, lupus, and the like. Pharmaceutical compositions comprising AGP-3-related proteins and AGP-3 agonists and antagonists are also encompassed by the invention.

In addition to therapeutic applications, AGP-3 related proteins may also be useful in production of hybridoma cells, which are derived from B cells. Thus, the present invention also concerns a method to modulate hybridoma cell antibody production, which comprises treating hybridoma cells with the compound of Claim 1.

Description of the Figures

Figure 1 shows the sequence of human AGP-3. Nucleic acid and amino acid sequences of human AGP-3 are indicated (SEQ ID NOS: 1 and

2, respectively). The predicted transmembrane region is underlined. Potential N-linked glycosylation sites are shown in boldface.

Figure 2 shows the sequence of murine AGP-3. Nucleic acid and amino acid sequences of murine AGP-3 are indicated (SEQ ID NOS: 3 and 4, respectively). The predicted transmembrane region is underlined. Potential N-linked glycosylation sites are shown in boldface.

Figure 3 shows an alignment of human and murine AGP-3, along with a consensus sequence (SEQ ID NO: 5). The predicted human and murine AGP-3 protein sequences were aligned by Pileup with gap creation penalty (12) and gap extension penalty (4) (Wisconsin GCG Package, Version 8.1, Genetics Computer Group Inc., Madison, Wisconsin). The consensus sequence was determined by Lineup (Wisconsin GCG Package, Version 8.1). The transmembrane regions from amino acid 47 to 72 in human AGP-3 and from amino acid 48 to 73 in murine AGP-3 are underlined. The N-terminal intracellular domain resides from amino acid 1 to 46 in human AGP-3 and from amino acid 1 to 47 in murine AGP-3. The C-terminal extracellular domain is localized from amino acid 73 to 285 in human AGP-3, and from amino acid 74 to 309. The human and murine AGP-3 share 68% amino acid identity overall. The C-terminus of AGP-3 is more conserved between human and mouse, with 87% identity over a 142-amino acid length. The putative conserved beta strands are indicated at the top, with the amino acids forming the putative strands underlined.

Figure 4 shows human and murine AGP-3 mRNA tissue distribution. Human tissue northern blots (A) and murine tissue northern blots (B) were probed with ^{32}P-labeled human AGP-3 probe (A) or murine AGP-3 probe. The probed blots were exposed to Kodak film for 18 hours (A) or seven days (B).

Figure 5 shows histology analysis of AGP-3 transgenic mouse spleen. The spleen sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and exosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The spleen of the transgenic mouse was enlarged, mainly due to the increase of size and number of the follicles. The B cell staining areas in the spleen follicles in the transgenic mouse were enlarged. The T cell number was slightly diminished.

Figure 6 shows histology analysis of AGP-3 transgenic mouse lymph nodes. The lymph node sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and exosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The lymph node size of the transgenic mouse was enlarged. The B cell number was greatly increased in the transgenic mouse. Instead of restricted to marginal zones of the follicles as in the control mouse, the B cells also filled out the follicular area in the lymph nodes of the transgenic mouse. The T cell number was decreased in the transgenic mouse as compared to the control.

Figure 7 shows histology analysis of AGP-3 transgenic mouse Peyer's patches. The Peyer's patches sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and exosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The histologic and immunohistologic changes were similar to the changes in the lymph node of the transgenic mouse.

Figure 8 shows FACS analysis of thymocytes, splenocytes and lymph node cells from AGP-3 transgenic mouse. Single-cell suspensions were prepared from spleen, lymph nodes and thymus from 10 AGP-3 transgenic mice and 5 control littermates. Cells were stained with FITC or PE-conjugated monoclonal antibodies against Thy-1.2, B220, CD11b, Gr-1,

CD4 or CD8. The B cell population increased by 100% in the transgenic mice as compared to the control mice. The T cell population decreased approximately 36%, with similar reductions in both CD4+ and CD8+ populations. Similar changes, though to a lesser degree, were observed in splenocytes. No differences in thymocyte staining were observed between the transgenic or control group.

Figure 9 shows a sequence comparison of the C-terminal region of members of the TNF ligand family determined via Pileup (Wisconsin GCG Package, Version 8.1). Amino acid numbers are indicated on the left side. The putative conserved beta strands and loops are indicated at the top. The predicted N-glycosylation sites are indicated with asterisks. The top line shows the consensus sequence (SEQ ID NO: 6). The remaining lines show the sequence for the C-terminal region of the mammalian TNF-related protein identified (SEQ ID NOS: 7 to 24, 40)

Figure 10 shows histology analysis of AGP-3 transgenic mice. Sections of spleen (A, B, C), lymph node (D, E, F) and Payer's patches (G, H, I) from control mice (left panel) and AGP-3 transgenic mice (right panel) were stained with hematoxylin and exosin (A, D, and G), anti-mouse B220 antibody (B, E, and H), or anti-mouse CD3 antibody (C, F, and I). Stained sections were analyzed under microscope at 10x.

Figure 11 shows FACS analysis splenocytes, lymph node cells and thymocytes of AGP-3 transgenic mice. Single-cell suspensions were prepared form spleen, lymph nodes and thymus from 10 AGP-3 transgenic mice and 5 control littermates. Cells with stained with FITC or PE-conjugated monoclonal antibodies against thy-1.2, B220, CD11b, Gr-1, CD4 or CD8.

Figure 12 shows elevation of serum immunoglobulin levels in AGP-3 transgenic mice. Control mice (n=5) and AGP-3 transgenic mice (n=5) were bled successively at 6, 7, 8, 9, 11 and 12 weeks of age. Serum IgM,

IgG, IgA, and IgE levels were quantitated by ELISA. Values are expressed as Mean ± SEM. All AGP-3 immunoglobulin levels were significantly increased (T-test; $P < 0.05$) compared to control groups.

Figure 13 shows kidney immunoglobulin deposits in AGP-3 transgenic mice. Kidney sections of 5 month control littermate (A, B, C), 5 month old AGP-3 mice (D, E, F), and 8 month old AGP-3 mice (G, H, I) were stained hematoxylin and exosin (A, D, and G), anti-mouse IgM (B, E, and H), anti-mouse IgG (C, F, and I), and Trichrome (G insert) Stained sections were analyzed under microscope at 60x.

Figure 14 shows that AGP-3 stimulates B cell survival and proliferation.

A. Increased B cell viability in AGP-3 transgenic mice. B cells were isolated from spleens of 3 month old AGP-3 transgenic mice (n-3) and control littermates (n=3). A total of 2.5×10^5 B cells was aliquoted per well in a 96-well round bottom plate and incubated for 9 days. At the indicated days, cells were incubated with 5 µg/ml Propidium Iodide and subject to FACS analysis for positive staining cells. Values are expressed as Mean ± SEM.

B. AGP-3 stimulates B cell proliferation. Purified B cells (10^5) from B6 mice were cultured in triplicates in 96 well plate with indicated amount of AGP-3 at the absence (upper panel) or presence of 2 µg/ml anti-IgM antibody (lower panel) for a period of 4 days. Proliferation was measured by radioactive ^{3}(H) thymidine uptake in last 18 hours of pulse. Data shown represent mean ± standard deviation of triplicate wells.

Detailed Description of the Invention

Definition of Terms

The following definitions apply to the terms used throughout this specification, unless otherwise limited in specific instances.

The term "AGP-3 related protein" refers to natural and recombinant proteins comprising the following sequence:

QDCLQLIADSXTPTIXKGXYTFVPWLLSF

(SEQ ID NO: 25)

wherein "X" may be any naturally occurring amino acid residue. This sequence is a consensus of the B and B' β-sheets and B/B' loop of hAGP-3 and mAGP-3 (see Figure 3), which is believed to be the specific receptor binding site. Preferred AGP-3-related proteins comprise both the B/B' consensus and the E/F consensus:

AMGHXIQRKKVHVFGDELSLVTLFR

(SEQ ID NO: 26)

The E/F region is also believed to be involved in receptor binding. More preferred proteins are those comprising the consensus of the B-I region:

QDCLQLIADS XTPTIXKGXY TFVPWLLSFK RGXALEEKEN KIXVXXTGYF FIYXQVLYTD XXXAMGHXIQ RKKVHVFGDE LSLVTLFRCI QNMPXTLPNN SCYSAGIAXL EEGDEXQLAI PRENAQISXX GDXTFFGALK LL

(SEQ ID NO: 27)

"AGP-3-related activity" means that a natural or recombinant protein, analog, derivative or fragment is capable of modulating B cell growth, survival, or activation, particularly in MLN, spleen, and Peyer's patches. The inventors contemplate that some molecules of interest may have activity antagonistic to native AGP-3 activity; for example, a derivative or analog may retain AGP-3 binding activity but will not activate the AGP-3 receptor. All such activity (agonism and antagonism of AGP-3) falls within the meaning of "AGP-3 related activity." Such activity can be determined, for example, by such assays as described in "Biological activity of AGP-3" in the Materials & Methods hereinafter, which may be modified as needed by many methods known to persons having ordinary skill in the art.

An "analog" of an AGP-3 protein (e.g., hAGP-3) is a polypeptide within the definition of "AGP-3-related protein" or "AGP-3-related protein," respectively, that has a substitution or addition of one or more amino acids. Such an AGP-3-related protein should maintain the property of eliciting B cell growth, survival, or activation. Such analogs will have substitutions or additions at any place along the polypeptide. Preferred analogs include those of soluble AGP-3-related proteins. Fragments or analogs may be naturally occurring, such as a polypeptide product of an allelic variant or a mRNA splice variant, or they may be constructed using techniques available to one skilled in the art for manipulating and synthesizing nucleic acids. The polypeptides may or may not have an amino terminal methionine residue.

A "derivative" of an AGP-3 protein is a polypeptide within the definition of "AGP-3-related protein" that has undergone post-translational modifications. Such modifications include, for example, addition of N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends, attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of an N-terminal methionine residue due to prokaryotic host cell expression. In particular, chemically modified derivatives of AGP-3-related protein that provide additional advantages such as increased stability, longer circulating time, or decreased immunogenicity are contemplated. Of particular use is modification with water soluble polymers, such as polyethylene glycol and derivatives thereof (see for example U.S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule,

or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties. Polypeptides may also be modified at pre-determined positions in the polypeptide, such as at the amino terminus, or at a selected lysine or arginine residue within the polypeptide. Other chemical modifications provided include a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

The term "protein" refers to polypeptides regardless of length or origin, comprising molecules that are recombinantly produced or naturally occurring, full length or truncated, having a natural sequence or mutated sequence, with or without post-translational modification, whether produced in mammalian cells, bacterial cells, or any other expression system.

The invention provides for proteins referred to as AGP-3 protein, or AGP-3-related proteins that primarily act on B cells. An EST bearing a portion of the AGP-3 sequence was obtained from a human fetal liver spleen cDNA library. A labeled cDNA fragment was used to probe a human spleen cDNA phage library (see "Cloning of Human AGP-3" in Materials & Methods hereinafter). The cDNA encoding a human AGP-3 was isolated from this phage library. The human protein is a type II transmembrane protein, having a short N-terminal intracellular region that differed from other members of the TNF ligand family and a long C-terminal extracellular region that comprises most of the conserved region of the TNF ligand family.

An EST encoding a murine ortholog was identified by BLAST search of Genebank using the human AGP-3 sequence. The corresponding cDNA clone was obtained from a mouse lymph node library and used to probe a mouse spleen cDNA phage library (see Materials & Methods

hereinafter). The cDNA encoding a murine AGP-3 ortholog was isolated from this phage library.

Northern blots were used to determine tissue distribution of transcription of AGP-3 (see "Cloning of Murine AGP-3" in Materials & Methods hereinafter). In murine tissue, AGP-3 mRNA was detected mainly in spleen, lung, liver, and kidney. In human tissue, AGP-3 mRNA was detected predominantly in peripheral blood leukocytes, with weaker transcription in spleen, lung, and small intestine (see Figures 4A and 4B).

The murine ortholog of AGP-3 was overexpressed in transgenic mice (see "Overexpression of murine AGP-3 in transgenic mice" in Materials & Methods hereinafter). In these transgenic mice, serum globulin and total protein levels increased greatly over control littermates while the albumin level remained the same (see "Biological Activity of AGP-3" in Materials & Methods hereinafter). The mice also exhibited increases in the size and number of follicles in the spleen, lymph nodes, and Peyer's patches (Figures 5, 6, and 7). In their MLN, the mice exhibited 100% increases in the number of cells expressing CD45 receptor with concomitant decreases in cells expressing CD90, CD4, and CD8. These results correspond to an increase in the B cell population and a decrease in the T cell population in the MLN (Figures 6 and 8). Similar results were obtained in the spleen, but to a lesser extent (Figures 5 and 8).

Nucleic Acids

The invention provides for isolated nucleic acids encoding AGP-3-related proteins. As used herein, the term "nucleic acid" comprises cDNA, genomic DNA, wholly or partially synthetic DNA, and RNA. These nucleic acids may be prepared or isolated as described in the working examples hereinafter or by nucleic acid hybridization thereof.

Nucleic acid hybridization typically involves a multi-step process. A first hybridization step forms nucleic acid duplexes from single strands.

A second hybridization step under more stringent conditions selectively retains nucleic acid duplexes having the desired homology. The conditions of the first hybridization step are generally not crucial, provided they are not of higher stringency than the second hybridization step. Generally, the second hybridization is carried out under conditions of high stringency, wherein "high stringency" conditions refers to conditions of temperature and salt that are about 12-20 °C below the melting temperature (T_m) of a perfect hybrid of part or all of the complementary strands corresponding to Figure 1 (SEQ ID NO: 1) and Figure 2 (SEQ ID NO: 3). In one embodiment, "high stringency" conditions refer to conditions of about 65 °C and not more than about 1 M Na^+. It is understood that salt concentration, temperature and/or length of incubation may be varied in either the first or second hybridization steps such that one obtains the hybridizing nucleic acid molecules according to the invention. Conditions for hybridization of nucleic acids and calculations of T_m for nucleic acid hybrids are described in Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, New York.

The nucleic acids of the invention may hybridize to part or all of the polypeptide coding regions of AGP-3 related proteins (e.g., SEQ ID NOS: 2 and 4 as shown in Figures 1 and 2) and therefore may be truncations or extensions of the nucleic acid sequences shown therein. Truncated or extended nucleic acids are encompassed by the invention provided that the encoded proteins retain AGP-3 related activity. In one embodiment, the nucleic acid will encode a polypeptide of at least about 10 amino acids. In another embodiment, the nucleic acid will encode a polypeptide of at least about 20 amino acids. In yet another embodiment, the nucleic acid will encode a polypeptide of at least about 50 amino acids. The hybridizing nucleic acids may also include noncoding sequences located 5' and/or 3' to the coding regions for the AGP-3 related protein. Noncoding

sequences include regulatory regions involved in expression of AGP-3 related protein, such as promoters, enhancer regions, translational initiation sites, transcription termination sites and the like.

In preferred embodiments, the nucleic acids of the invention encode mouse or human AGP-3. Most preferred are the nucleic acids encoding proteins of SEQ ID NOS: 25, 26, or 27. Nucleic acids may encode a membrane-bound form of AGP-3-related protein or soluble forms. For human AGP-3-related protein, the predicted transmembrane region includes amino acid residues 47-72 inclusive as shown in Figure 1 (SEQ. ID. NO: 2); for murine AGP-3 related protein, residues 48-73 inclusive as shown in Figure 2 (SEQ ID NO: 4). Substitutions that replace hydrophobic amino acid residues in this region with neutral or hydrophilic amino acid residues would be expected to disrupt membrane association and result in soluble AGP-3-related protein. In addition, deletions of part or all the transmembrane region would also be expected to produce soluble forms of AGP-3-related protein. Nucleic acids encoding SEQ ID NO: 5 as shown in Figure 3 or fragments and analogs thereof, encompass soluble AGP-3-related proteins.

Nucleic acid sequences of the invention may also be used for the detection of sequences encoding AGP-3-related protein in biological samples. In particular, the sequences may be used to screen cDNA and genomic libraries for related AGP-3-related protein sequences, especially those from other species. The nucleic acids are also useful for modulating levels of AGP-3-related protein by anti-sense technology or in vivo gene expression. Development of transgenic animals expressing AGP-3-related protein are useful for production of the polypeptides and for the study of in vivo biological activity.

Vectors and Host Cells

The nucleic acids of the invention will be linked with DNA sequences so as to express biologically active AGP-3-related protein. Sequences required for expression are known to those skilled in the art and include promoters and enhancer sequences for initiation of RNA synthesis, transcription termination sites, ribosome binding sites for the initiation of protein synthesis, and leader sequences for secretion. Sequences directing expression and secretion of AGP-3-related protein may be homologous, i.e., the sequences are identical or similar to those sequences in the genome involved in AGP-3-related protein expression and secretion, or they may be heterologous. A variety of plasmid vectors are available for expressing AGP-3-related protein in host cells (see, for example, Methods in Enzymology v. 185, Goeddel, D.V. ed., Academic Press (1990)). For expression in mammalian host cells, a preferred embodiment is plasmid pDSRα described in PCT Application No. 90/14363. For expression in bacterial host cells, preferred embodiments include plasmids harboring the lux promoter (see co-owned and co-pending U.S. Serial No. 08/577,778, filed December 22, 1995). In addition, vectors are available for the tissue-specific expression of AGP-3-related protein in transgenic animals. Gene transfer vectors derived from retrovirus (RV), adenovirus (AdV), and adeno-associated virus (AAV) may also be used for the expression of AGP-3 related protein in human cells for in vivo therapy (see PCT Application No. 86/00922).

Prokaryotic and eukaryotic host cells expressing AGP-3-related protein are also provided by the invention. Host cells include bacterial, yeast, plant, insect or mammalian cells. AGP-3-related protein may also be produced in transgenic animals, such as mice or goats. Plasmids and vectors containing the nucleic acids of the invention are introduced into appropriate host cells using transfection or transformation techniques known to one skilled in the art. Host cells may contain DNA sequences

encoding AGP-3-related protein as shown in Figures 1, 2, or 3, or a portion of either thereof, such as the extracellular domain or the cytoplasmic domain. Nucleic acids encoding AGP-3-related proteins may be modified by substitution of codons that allow for optimal expression in a given host. At least some of the codons may be so-called preference codons that do not alter the amino acid sequence and are frequently found in genes that are highly expressed. However, it is understood that codon alterations to optimize expression are not restricted to the introduction of preference codons. Examples of preferred mammalian host cells for AGP-3-related protein expression include, but are not limited to COS, CHOd-, 293 and 3T3 cells. A preferred bacterial host cell is Escherichia coli.

Polypeptides

The invention also provides AGP-3-related proteins as the products of prokaryotic or eukaryotic expression of exogenous DNA sequences. Exogenous DNA sequences include cDNA, genomic DNA and synthetic DNA sequences. AGP-3-related proteins may be the products of bacterial, yeast, plant, insect or mammalian cells expression, or from cell-free translation systems. AGP-3-related proteins produced in bacterial cells will have N-terminal methionine residues. The invention also provides for a process of producing AGP-3-related proteins comprising growing prokaryotic or eukaryotic host cells transformed or transfected with nucleic acids encoding them and isolating polypeptide expression products of the nucleic acids.

Polypeptides that are mammalian proteins or are fragments, analogs or derivatives thereof are encompassed by the invention. In preferred embodiments, the AGP-3-related protein is human AGP-3 protein. A fragment of AGP-3-related protein refers to a polypeptide having a deletion of one or more amino acids such that the resulting polypeptide retains AGP-3 related activity; for example, the polypeptide

has at least the property of eliciting or antagonizing B cell growth, survival, or activation, especially in mesenteric lymph nodes. Said fragments will have deletions originating from the amino terminal end, the carboxy terminal end, and internal regions of the polypeptide. Fragments of AGP-3-related proteins are at least about ten amino acids, at least about 20 amino acids, or at least about 50 amino acids in length. In preferred embodiments, AGP-3-related proteins will have a deletion of one or more amino acids from the transmembrane region (amino acid residues 48-73 as shown in Figure 1), or, alternatively, one or more amino acids from the amino-terminus up to and/or including the transmembrane region (amino acid residues 1-73 as shown in Figure 1). Such polypeptides may act as agonists or antagonists of the ligand:receptor interaction and activate or inhibit ligand-mediated activity of AGP-3 related protein. Such antagonists and/or agonists can be examined for AGP-3 related activity (see "Biological activity of AGP-3" in Materials & Methods hereinafter).

The polypeptides of the invention are isolated and purified from tissues and cell lines that express AGP-3 related protein, either extracted from lysates or from conditioned growth medium, and from transformed host cells expressing AGP-3 related protein. Human AGP-3 related protein, or nucleic acids encoding same, may be isolated from human lymph node or fetal liver tissue. Isolated AGP-3 related protein is free from association with human proteins and other cell constituents.

A method for purification of such proteins from natural sources (e.g. tissues and cell lines that normally express an AGP-3 related protein) and from transfected host cells is also encompassed by the invention. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The chromatography steps can include ion exchange, gel filtration,

hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-AGP-3-related protein antibody or biotin-streptavidin affinity complex and the like.

Fusion proteins and derivatives

The invention further comprises AGP-3-related protein chimeras, as well as such proteins derivatized by linkage to such molecules as PEG or dextran. Such proteins comprise part or all of an AGP-3-related protein amino acid sequence fused to a heterologous amino acid sequence. The heterologous sequence may be any sequence that allows the resulting fusion protein to retain AGP-3-related activity (i.e., AGP-3 agonists) or will maintain AGP-3 binding activity but not have AGP-3 related activity as defined herein (i.e., AGP-3 antagonists). Such fragments, derivatives or analogs of AGP-3 can be examined for their ability to agonize or antagonize AGP-3-mediated B cell growth, survival, or activation associated with the disease or condition (see "Biological activity of AGP-3" in Materials & Methods hereinafter). In preferred embodiments, a heterologous sequence is fused to a sequence comprising an AGP-3 related protein's B/B' region (SEQ ID NO: 25) and/or the E/F region (SEQ ID NO: 26) or to the more complete B-I region (SEQ ID NO: 27). Such heterologous sequences include cytoplasmic domains that allow for alternative intracellular signaling events, sequences that promote oligomerization (e.g., the Fc region of IgG), enzyme sequences that provide a label for the polypeptide, and sequences that provide affinity probes (e.g., an antigen-antibody recognition site).

Preferred molecules in accordance with this invention are Fc-linked AGP-3 related proteins. Useful modifications of protein therapeutic agents by fusion with the "Fc" domain of an antibody are discussed in detail in a patent application entitled, "Modified Peptides as Therapeutic Agents," U.S. Ser. No. 09/428,082, PCT appl. no. WO 99/25044, which is hereby

incorporated by reference in its entirety. That patent application discusses linkage to a "vehicle" such as PEG, dextran, or an Fc region.

In the compositions of matter prepared in accordance with this invention, the AGP-3 related protein may be attached to a vehicle through the protein's N-terminus or C-terminus. Thus, the vehicle-protein molecules of this invention may be described by the following formula I:

I

$$(X^1)_a\text{-}F^1\text{-}(X^2)_b$$

wherein:

F^1 is a vehicle (preferably an Fc domain);

X^1 and X^2 are each independently selected from $-(L^1)_c\text{-}P^1$, $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2$, $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3$, and $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3\text{-}(L^4)_f\text{-}P^4$

P^1, P^2, P^3, and P^4 are each independently sequences of AGP-3 related protein (e.g., a fragment of hAGP-3);

L^1, L^2, L^3, and L^4 are each independently linkers; and

a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1.

Thus, compound I comprises preferred compounds of the formulae

II

$$X^1\text{-}F^1$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the C-terminus of X^1;

III

$$F^1\text{-}X^2$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the N-terminus of X^2;

IV

$$F^1\text{-}(L^1)_c\text{-}P^1$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the N-terminus of $-(L^1)_c-P^1$; and

V

$$F^1-(L^1)_c-P^1-(L^2)_d-P^2$$

and multimers thereof wherein F^1 is an Fc domain and is attached at the N-terminus of $-L^1-P^1-L^2-P^2$.

Antibodies

Uses for antibodies specifically binding the polypeptides of the invention are also encompassed by the invention. The antibodies may be generated by immunization with full-length AGP-3 related protein, or fragments thereof. Preferred antibodies bind to SEQ ID NOS: 25, 26, or 27. Such antibodies may be generated by immunization with polypeptides comprising those sequences. The term "antibodies" also refers to molecules having Fv, Fc and other structural domains usually associated with antibodies but that may be generated by other techniques (e.g., phage display antibody generation). The antibodies of the invention may be polyclonal or monoclonal, or may be recombinant antibodies, such as chimeric antibodies wherein the murine constant regions on light and heavy chains are replaced by human sequences, or CDR-grafted antibodies wherein only the complementarity determining regions are of murine origin. Antibodies of the invention may also be fully human antibodies prepared, for example, by immunization of transgenic animals capable of producing human antibodies (see, for example, PCT Application No. WO93/12227). Regardless of the means by which they are generated, antibodies in accordance with this invention may be produced by recombinant means (e.g., transfection of CHO cells with vectors comprising antibody sequence).

The antibodies are useful for detecting AGP-3 related protein in biological samples, thereby allowing the identification of cells or tissues

that produce such proteins. In addition, antibodies that bind to AGP-3 related proteins and block interaction with other binding compounds (i.e., "antagonist antibodies") have therapeutic use in modulating B cell growth, activation, and/or proliferation. Antibodies can be tested for binding to AGP-3 related protein and examined for their effect on AGP-3-mediated B cell growth, survival, or activation associated with the disease or condition (see "Biological activity of AGP-3" in Materials & Methods hereinafter).

Compositions

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the AGP-3 related protein of the invention together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of an AGP-3 related protein agonist or antagonist. The term "therapeutically effective amount" means an amount that provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form and comprises one or more of the following:

- a diluent (e.g., Tris, acetate or phosphate buffers) having various pH values and ionic strengths;
- a solubilizer (e.g., Tween or Polysorbate);
- carriers (e.g., human serum albumin or gelatin);
- preservatives (e.g., thimerosal or benzyl alcohol); and
- antioxidants (e.g., ascorbic acid or sodium metabisulfite).

Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive survey of component suitable for pharmaceutical compositions is found in

Remington's Pharmaceutical Sciences (1980), 18th ed. (A. R. Gennaro, ed.) Mack, Easton, PA.

In a preferred embodiment, compositions comprising AGP-3 antibody or soluble AGP-3-related protein are provided. Also encompassed are compositions comprising soluble AGP-3-related protein modified with water-soluble polymers to increase solubility, stability, plasma half-life and bioavailability. Compositions may also comprise incorporation of soluble AGP-3 related protein into liposomes, microemulsions, micelles or vesicles for controlled delivery over an extended period of time. Soluble AGP-3 related protein may be formulated into microparticles suitable for pulmonary administration.

Compositions of the invention may be administered by injection (either subcutaneous, intravenous or intramuscular) or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one of ordinary skill in the art.

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the coding region of AGP-3 related protein and/or flanking regions to cells and tissues as part of an anti-sense therapy regimen.

Pharmaceutical Methods of Use

AGP-3 related proteins and agonists or antagonists thereof may be used to treat conditions characterized by B cell growth, survival, and activation, such as autoimmune and inflammatory disorders. The invention also encompasses modulators (agonists and antagonists) of AGP-3-related protein and methods for obtaining them. Such a modulator may either increase or decrease at least one activity associated with AGP-

3, such as B cell growth, survival, or activation in MLN, spleen, and Peyer's patches. Typically, an agonist or antagonist may be a co-factor, such as a protein, peptide, carbohydrate, lipid or small molecular weight molecule, that interacts with AGP-3 and regulates activity. Potential polypeptide antagonists include antibodies that react with soluble or membrane-associated forms of AGP-3, a fragment of AGP-3 (e.g., SEQ ID NO: 25) and an Fc-linked AGP-3 fragment. Molecules that regulate AGP-3-related protein expression typically include nucleic acids that are complementary to nucleic acids encoding AGP-3-related protein and that act as anti-sense regulators of expression.

AGP-3-related proteins and modulators thereof may be particularly useful in treatment of inflammatory conditions of the joints. Inflammatory conditions of a joint are chronic joint diseases that afflict and disable, to varying degrees, millions of people worldwide. Rheumatoid arthritis is a disease of articular joints in which the cartilage and bone are slowly eroded away by a proliferative, invasive connective tissue called pannus, which is derived from the synovial membrane. The disease may involve peri-articular structures such as bursae, tendon sheaths and tendons as well as extra-articular tissues such as the subcutis, cardiovascular system, lungs, spleen, lymph nodes, skeletal muscles, nervous system (central and peripheral) and eyes (Silberberg (1985), Anderson's Pathology, Kissane (ed.), II:1828). Osteoarthritis is a common joint disease characterized by degenerative changes in articular cartilage and reactive proliferation of bone and cartilage around the joint. Osteoarthritis is a cell-mediated active process that may result from the inappropriate response of chondrocytes to catabolic and anabolic stimuli. Changes in some matrix molecules of articular cartilage reportedly occur in early osteoarthritis (Thonar et al. (1993), Rheumatic disease clinics of North America, Moskowitz (ed.), 19:635-657 and Shinmei et al. (1992), Arthritis Rheum.,

35:1304-1308). AGP-3, AGP-3 R and modulators thereof are believed to be useful in the treatment of these and related conditions.

AGP-3 related proteins and agonists or antagonists thereof may also be useful in treatment of a number of additional diseases and disorders, including:

acute pancreatitis;

ALS;

Alzheimer's disease;

asthma;

atherosclerosis;

cachexia/anorexia;

chronic fatigue syndrome;

diabetes (e.g., insulin diabetes);

fever;

glomerulonephritis;

graft versus host disease;

hemorrhagic shock;

hyperalgesia;

inflammatory bowel disease;

inflammatory conditions of a joint, including osteoarthritis, psoriatic arthritis and rheumatoid arthritis;

inflammatory conditions resulting from strain, sprain, cartilage damage, trauma, orthopedic surgery, infection or other disease processes;

ischemic injury, including cerebral ischemia (e.g., brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration);

learning impairment;

lung diseases (e.g., ARDS);

multiple myeloma;

multiple sclerosis;
myelogenous leukemia (e.g., AML and CML) and other leukemias;
myopathies (e.g., muscle protein metabolism, esp. in sepsis);
neurotoxicity (e.g., as induced by HIV);
osteoporosis;
pain;
Parkinson's disease;
pre-term labor;
psoriasis;
reperfusion injury;
septic shock;
side effects from radiation therapy;
sleep disturbance;
temporal mandibular joint disease; and
tumor metastasis.

Agonists and antagonists of AGP-3-related protein may be administered alone or in combination with a therapeutically effective amount of other drugs, including analgesic agents, disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and any immune and/or inflammatory modulators. Thus, agonists and antagonists of AGP-3 related protein may be administered with:

- Modulators of other members of the TNF/TNF receptor family, including TNF antagonists, such as etanercept (Enbrel™), sTNF-RI, D2E7, and Remicade™.
- Nerve growth factor (NGF) modulators.
- IL-1 inhibitors, including IL-1ra molecules such as anakinra (Kineret™) and more recently discovered IL-1ra-like molecules such as IL-1Hy1 and IL-1Hy2; IL-1 "trap" molecules as described

in U.S. Pat. No. 5,844,099, issued December 1, 1998; IL-1 antibodies; solubilized IL-1 receptor, and the like.

- IL-6 inhibitors (e.g., antibodies to IL-6).
- IL-8 inhibitors (e.g., antibodies to IL-8).
- IL-18 inhibitors (e.g., IL-18 binding protein, solubilized IL-18 receptor, or IL-18 antibodies).
- Interleukin-1 converting enzyme (ICE) modulators.
- insulin-like growth factors (IGF-1, IGF-2) and modulators thereof.
- Transforming growth factor-β (TGF-β), TGF-β family members, and TGF-β modulators.
- Fibroblast growth factors FGF-1 to FGF-10, and FGF modulators.
- Osteoprotegerin (OPG), OPG analogues, osteoprotective agents, and bone anabolic agents.
- PAF antagonists.
- Keratinocyte growth factor (KGF), KGF-related molecules (e.g., KGF-2), and KGF modulators.
- COX-2 inhibitors, such as Celebrex™ and Vioxx™.
- Prostaglandin analogs (e.g., E series prostaglandins).
- Matrix metalloproteinase (MMP) modulators.
- Nitric oxide synthase (NOS) modulators, including modulators of inducible NOS.
- Modulators of glucocorticoid receptor.
- Modulators of glutamate receptor.
- Modulators of lipopolysaccharide (LPS) levels.
- Anti-cancer agents, including inhibitors of oncogenes (e.g., fos, jun) and interferons.
- Noradrenaline and modulators and mimetics thereof.

Assay Methods of Use

AGP-3-related proteins may be used in a variety of assays for detecting agonists, antagonists and characterizing interactions with AGP-3 related proteins. In general, the assay comprises incubating AGP-3-related protein under conditions that permit measurement of AGP-3-related activity as defined above. Qualitative or quantitative assays may be developed. Assays may also be used to identify new AGP-3 agonists or antagonists and AGP-3 related potein family members.

Binding assays for agonists, or antagonists to natural or recombinant AGP-3 related protein may be carried out in several formats, including cell-based binding assays, membrane binding assays, solution-phase assays and immunoassays. In general, trace levels of a labeled binding molecule are incubated with AGP-3-related protein samples for a specified period of time followed by measurement of bound molecule by filtration, electrochemiluminescent (ECL, ORIGEN system by IGEN), cell-based or immunoassays. Homogeneous assay technologies for radioactivity (SPA; Amersham) and time-resolved fluoresence (HTRF, Packard) can also be implemented. Binding is detected by labeling a binding molecule (e.g., an anti-AGP-3 antibody) with radioactive isotopes (125I, 35S, 3H), fluorescent dyes (fluorescein), lanthanide (Eu^{3+}) chelates or cryptates, orbipyridyl-ruthenium (Ru^{2+}) complexes. It is understood that the choice of a labeled probe will depend upon the detection system used. Alternatively, a binding molecule may be modified with an unlabeled epitope tag (e.g., biotin, peptides, His_6, myc) and bound to proteins such as streptavidin, anti-peptide or anti-protein antibodies that have a detectable label as described above.

Binding molecules in such assays may be nucleic acids, proteins, peptides, carbohydrates, lipids or small molecular weight organic compounds. The binding molecule may be substantially purified or

present in a crude mixture. The binding molecules may be further characterized by their ability to increase or decrease AGP-3 related activity in order to determine whether they act as an agonist or an antagonist.

In an alternative method, AGP-3-related protein may be assayed directly using polyclonal or monoclonal antibodies to AGP-3 related proteins in an immunoassay. Additional forms of AGP-3-related proteins containing epitope tags as described above may be used in solution and immunoassays.

AGP-3 related proteins are also useful for identification of intracellular proteins that interact with their respective cytoplasmic domains by a yeast two-hybrid screening process. As an example, hybrid constructs comprising DNA encoding the N-terminal 50 amino acids of an AGP-3 related protein fused to a yeast GAL4-DNA binding domain may be used as a two-hybrid bait plasmid. Positive clones emerging from the screening may be characterized further to identify interacting proteins. This information may help elucidate an intracellular signaling mechanism associated with AGP-3-related activity and provide intracellular targets for new drugs that modulate inflammatory and immune-related diseases and conditions.

A variety of assays may be used to measure the interaction of AGP-3-related proteins and agonists, antagonists, or other ligands in vitro using purified proteins. These assays may be used to screen compounds for their ability to increase or decrease the rate or extent of binding to AGP-3 related proteins. In one type of assay, AGP-3 related protein can be immobilized by attachment to the bottom of the wells of a microtiter plate. A radiolabeled binding molecule and a test molecule can then be added either one at a time (in either order) or simultaneously to the wells. After incubation, the wells can be washed and counted using a scintillation counter for radioactivity to determine the extent of binding to AGP-3

related protein. Typically, molecules will be tested over a range of concentrations, and a series of control wells lacking one or more elements of the test assays can be used for accuracy in evaluation of the results. An alternative to this method involves reversing the "positions" of the proteins; i.e., immobilizing a binding molecule to the mictrotiter plate wells, incubating with the test compound and radiolabeled AGP-3 related protein, and determining the extent of binding. See, for example, chapter 18 of Current Protocols in Molecular Biology (1995) (Ausubel et al., eds.), John Wiley & Sons, New York, NY.

As an alternative to radiolabeling, AGP-3 related proteins or a binding molecule may be conjugated to biotin and the presence of biotinylated protein can then be detected using streptavidin linked to an enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), that can be detected colorimetrically, or by fluorescent tagging of streptavidin. An antibody directed to AGP-3 related protein or a binding molecule that is conjugated to biotin may also be used and can be detected after incubation with enzyme-linked streptavidin linked to AP or HRP.

AGP-3-related proteins or binding molecules may also be immobilized by attachment to agarose beads, acrylic beads or other types of such inert substrates. The substrate-protein complex can be placed in a solution containing the complementary protein and the test compound; after incubation, the beads can be precipitated by centrifugation, and the amount of binding between the AGP-3-related protein and a binding molecule can be assessed using the methods described above. Alternatively, the substrate-protein complex can be immobilized in a column and the test molecule and complementary molecule passed over the column. Formation of a complex between AGP-3 related protein and the binding molecule can then be assessed using any of the techniques set forth above (i.e., radiolabeling, antibody binding, and the like).

Another useful in vitro assay is a surface plasmon resonance detector system, such as the Biacore assay system (Pharmacia, Piscataway, NJ). The Biacore system may be carried out using the manufacturer's protocol. This assay essentially involves covalent binding of either an AGP-3 related protein or a binding molecule to a dextran-coated sensor chip that is located in a detector. The test compound and the other complementary protein can then be injected into the chamber containing the sensor chip either simultaneously or sequentially and the amount of complementary protein that binds can be assessed based on the change in molecular mass that is physically associated with the dextran-coated side of the of the sensor chip; the change in molecular mass can be measured by the detector system.

In vitro assays such as those described above may be used advantageously to screen rapidly large numbers of compounds for effects on complex formation with AGP-3-related proteins. The assays may be automated to screen compounds generated in phage display, synthetic peptide and chemical synthesis libraries.

Compounds that increase or decrease complex formation of AGP-3-related proteins and binding molecules may also be screened in cell culture using cells and cell lines bearing such ligands. Cells and cell lines may be obtained from any mammal, but preferably will be from human or other primate, canine, or rodent sources. Such cells may be enriched from other cell types by affinity chromatography using publicly available procedures. Attachment of AGP-3-related protein to such cells is evaluated in the presence or absence of test compounds and the extent of binding may be determined by, for example, flow cytometry using a biotinylated antibody. Cell culture assays may be used advantageously to further evaluate compounds that score positive in protein binding assays described above.

Description of Preferred Embodiments

The following examples are offered to illustrate the invention, but should not be construed as limiting the scope thereof.

Materials and Methods

Cloning of Human AGP-3

A TNF family profile search of the Genbank dbEST data base was performed. Smith et al.(1994), Cell, 76: 959-62; Luethy et al.(1994), Protein Science, 3: 139-46. One human EST sequence (GenBank accession number T87299) was identified as a possible new member of the TNF ligand. The EST was obtained from human fetal liver spleen cDNA library (The WashU-Merck EST Project). The cDNA clone (115371 3') corresponding to the EST sequence was obtained from Genome Systems, Inc. (St. Louis, MO). The cDNA fragment was released from the pT7T3D vector with EcoRI and NotI digestion. The fragment was approximately 0.7 kb in length and was used for the subsequent full-length cloning.

The ^{32}P-dCTP-labeled T87299 cDNA fragment was used as a probe to screen a human spleen cDNA phage library (Stratagene, La Jolla, CA). Recombinant phages were plated onto E. coli strain XL1-blue at approximately 5 x 10^4 transformants per 150 mm LB plate. Nitrocellulose filters were lifted from these plates in duplicates. Filters were prehybridized in 5x SSC, 50% deionized formamide, 5x Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA for 2 hours at 42 °C. The filters were then hybridized in the same solution with the addition of 5 ng/ml of labeled probe at 42°C overnight. The filters were first washed in 2x SSC and 0.1% SDS for 10 minutes at RT twice, and then washed in 0.1x SSC and 0.1% SDS at 65 °C for 30 minutes twice. The filters were then exposed to autoradiography with intensifying screens at -80 °C overnight. Positive hybridizing plaques were determined by aligning the duplicate filters, and then picked up for subsequent

secondary or tertiary screening till single isolated positive plaque was obtained. From total of one million recombinant phage clones, 8 positive plaques were obtained.

The pBluescript phagemid was excised from phage using the ExAssist™/SOLR™ System according to the manufacturer's description (Stratagene, La Jolla, CA). The excised phagemids were plated onto freshly grown SOLR cells on LB/ampicillin plates and incubated overnight. Single bacteria colony was amplified in LB media containing 100 µg/ml ampicillin. The plasmid DNA was prepared and both strands of cDNA insert were sequenced.

The human AGP-3 cDNA (clone 13-2) is 1.1 kb in length. It encodes a LORF of 285 amino acids. FASTA search of the SwissProt database with the predicted AGP-3 protein sequence indicated that it is mostly related to human TNFα with 25% identity in C-terminal 116 amino acid overlap. Like other TNF ligand family members, human AGP-3 protein is a type II transmembrane protein, containing a short N-terminal intracellular domain (amino acids 1-46), a hydrophobic transmembrane region (amino acids 47-68) following by a long C-terminal extracellular domain (amino acid 69-285). The C-terminal extracellular domain of AGP-3 contained most of the conserved region of the TNF ligand family. Smith et al.(1994), Cell, 76: 959-62.

Cloning of Murine AGP-3

An EST sequence (Genebank accession number AA254047) encoding a potential murine AGP-3 ortholog was identified by BLAST search of Genebank dbEST database with human AGP-3 sequence. The corresponding cDNA clone (722549 5') from mouse lymph node library was obtained from Genome Systems, Inc. (St. Louis, MO). The clone contained a 0.9 kb cDNA insert which could be released by EcoRI and NotI digestion. The 0.9 kb cDNA fragment encodes an open reading frame

of 96 amino acids which shares 87% identity with the corresponding C-terminal human AGP-3 polypeptide sequence. A 0.41 kb EcoRI-XmnI fragment, which contained 290 bp coding region and 120 bp 3' non-coding region, was used as probe to screening a mouse spleen cDNA phage library (Stratagene, La Jolla, CA) for full length murine AGP-3 cDNA as described above. From one million recombinant phage clones, 6 positive plaques were obtained. The phagemid was excised from phage as described above. The plasmid DNA was prepared and both strands of cDNA insert were sequenced. The murine AGP-3 cDNA (clone S6) encodes a polypeptide of 309 amino acids. Like its human ortholog, murine AGP-3 is also a type II transmembrane region, containing a short N-terminal intracellular domain (amino acid 1-46), a hydrophobic transmembrane region (amino acid 47-68) following by a long C-terminal extracellular domain (amino acid 69-285). The human and murine AGP-3 share 68% amino acid sequence identity overall. However, the C-terminal 142 amino acid sequences share 87% identity between the two species. Preceding the highly conserved C-terminus region, there is an insertion of 30 extra amino acids in the murine AGP-3. Four out of 7 positive phage plaques were independent clones, yet they all shared the same coding sequences.

Expression of human and murine AGP-3 mRNA

Multiple human or murine tissue northern blots (Clontech, Palo Alto, CA) were probed with ^{32}P-dCTP labeled human AGP-3 0.7kb EcoRI-NotI fragment or murine AGP-3 0.41kb EcoRI-XmnI fragment, respectively. The Northern blots were prehybridized in 5x SSC, 50% deionized formamide, 5xDenhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA for 2 hours at 42 °C. The blots were then hybridized in the same solution with the addition of 5ng/ml of labeled probe at 42°C overnight. The filters were first washed in 2x SSC and 0.1%

SDS for 10 minutes at RT twice, and then washed in 0.1x SSC and 0.1% SDS at 65 °C for 30 minutes twice. The blots were then exposed to autoradiography. The human tissue northern blot analysis with human AGP-3 probe under stringent conditions revealed predominant AGP-3 transcripts with a related molecular mass of 2.4kb in peripheral blood leukocytes (Figure 4A). Weaker expression was also detected in human spleen, lung and small intestine (Figure 4A). Among murine tissues analyzed, murine AGP-3 mRNA, with a relative molecular mass of 2kb, was mainly detected in spleen, lung, liver and kidney (Figure 4B).

Overexpression of murine AGP-3 in transgenic mice

Murine AGP-3 cDNA clone S6 in pBluescript SK(-) in pBluescript was used as template to PCR the entire coding region. T3 primer

5′ AAT TAA CCC TCA CTA AAG GG 3″

SEQ ID NO: 28

was used as 5′ PCR primer. The 3′ end PCR primer, which contained a XhoI site, was

5′ TCT CCC TCG AGA TCA CGC ACT CCA GCA AGT GAG 3′

SEQ ID NO: 29

PCR reactions were carried in a volume of 50 µl with 1 unit of vent DNA polymerase (New England Biolabs) in 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.1% Triton-X100, 10 µM of each dNTP, 1 µM of each primer and 10 ng of murine AGP-3 cDNA template. Reactions were performed in 94 °C for 45 s, 55 °C for 55 S, and 72 °C for 2 minutes, for a total of 35 cycles. The PCR fragment created a XhoI site at 3′ end after the AGP-3 coding region. The 1 kb PCR fragment was purified by electrophoresis, and digested with XbaI (present in the pBluescript MCS, 80 bp upstream of AGP-3 starting Methione) and XhoI restriction enzymes. The XbaI-XhoI PCR fragment was cloned into expression vector under the control of the human β-actin promoter. Graham et al.(1997), Nature Genetics 17: 272-4;

Ray et al.(1991), Genes Dev. 5: 2265-73. The PCR fragment was sequenced to ensure no mutation. The murine AGP-3 expression plasmid was purified through two rounds of CsCl density gradient centrifugation. The purified plasmid was digested with ClaI, and a 6 kb fragment containing murine AGP-3 transgene was purified by gel electrophoresis. The purified fragment was resuspended in 5 mM Tris, pH 7.4, 0.2 mM EDTA at 2 µg/ml concentration. Single-cell embryos from BDF1 x BDF1-bred mice were injected as described (WO97 /23614). Embryos were cultured overnight in a CO_2 incubator and 15-20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

Following term pregnancy, 62 offspring were obtained from implantation of microinjected embryos. The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. Ear pieces were digested in 20 µl ear buffer (20mM Tris, pH8.0, 10mM EDTA, 0.5% SDS, 500 µg/ml proteinase K) at 55°C overnight. The sample was diluted with 200 µl of TE, and 2 µl of the ear sample was used for the PCR reaction. The 5' PCR primer

5' AAC AGG CTA TTT CTT CAT CTA CAG 3'

SEQ ID NO: 30

resided in the murine AGP-3 coding region. The 3' PCR primer

5' CTC ATC AAT GTA TCT TAT CAT GTC T 3'

SEQ ID NO: 31

resided in the vector 3' to the murine AGP-3 transgene. The PCR reactions were carried in a volume of 50 µl with 0.5 unit of Tag DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 10 µM of each dNTP, 1 µM of each primer and 2 µl of ear sample. The mixtures were first heated at 94 °C for 2 min, and the PCR reactions were performed in 94 °C for 30 s, 55 °C for 30 s, and 72 °C

for 45 s, for a total of 35 cycles. Of the 62 offspring, 10 were identified as PCR positive transgenic founders.

At 8 weeks of age, all ten transgenic founders (animal 3, 6, 9, 10, 13, 38, 40, 58, 59, and 62) and five controls (animal 7, 8, 11, 12 and 14) were sacrificed for necropsy and pathological analysis. Portions of spleen were removed, and total cellular RNA was isolated from the spleens of all the transgenic founders and negative controls using the Total RNA Extraction Kit (Qiagen Inc., Chartsworth, CA). The expression of the transgene was determined by RT-PCR. The cDNA was synthesized using the SuperScript™ Preamplification System according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD). The primer

5′ CTC ATC AAT GTA TCT TAT CAT GTC T 3′

SEQ ID NO: 32

which was located in the expression vector sequence 3′ to the AGP-3 transgene, was used to prime cDNA synthesis from the transgene transcripts. Ten µg total spleen RNA from transgenic founders and controls were incubated with 1 µM of primer at 70°C for 10 min, and placed on ice. The reaction was then supplemented with 10 mM Tris-HCl pH 8.3, 50 mM KCL, 2.5 mM $MgCl_2$, 10 µM of each dNTP, 0.1 mM DTT and 200 U SuperScript II RT. After incubation at 42 °C for 50 min, the reaction was stopped by heating at 72 °C for 15 min. Total RNA were digested by addition of 2 U RNase H and incubation at 37 °C for 20 min. Subsequent PCR reactions were carried out by using murine AGP-3 specific primers. The 5′ PCR primer was

5′ AGC CGC GGC CAC AGG AAC AG 3′

SEQ ID NO: 33

The 3′ PCR primer was

5′ TGG ATG ACA TGA CCC ATA G 3′

SEQ ID NO: 34

The PCR reaction was performed in a volume of 50 µl with 0.5 unit Tag DNA polymerase in 10 mM Tris-HCl pH 8.3, 50 mM KCL, 2.5mM $MgCl_2$, 10 µM of each dNTP, 1 µM of each primer and 1 µl of cDNA product. The reaction was performed at 94 °C for 30 s, 55°C for 30 S, and 72 °C for 1 min, for a total of 35 cycles. The PCR product was analyzed by electrophoresis. Transgene expression was detected in the spleen of all ten AGP-3 transgenic mice founders.

Biological activity of AGP-3

Prior to euthanasia, all animals were weighed, anesthetized by isofluorane and blood was drawn by cardiac puncture. The samples were subjected to hematology and serum chemistry analysis. The serum globulin level in all the AGP-3 transgenic mice (animal 3, 6, 9, 10, 13, 38, 40, 58, 59 and 62) increased more than 100% as compared to the control littermates (animal 7, 8, 11, 12 and 14, Table 1). Total protein level also increased correspondingly in the transgenic group, while albumin level remained the same. No significant differences in other serum chemistry or hematology parameters were observed at this age.

Radiography was performed after terminal exsanguination. There was no difference in the radiodensity or radiologic morphology of the skeleton. Upon gross dissection, major visceral organs were subject to weight analysis. The spleen weight relative to the body weight increased by approximately 45% in the AGP-3 transgenic group as compared to the control mice. The sizes of lymph nodes and Peyer's patches were also increased substantially in all the AGP-3 transgenic mice.

Following gross dissection, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The tissues collected were liver, spleen, pancreas, stomach, the entire gastrointestinal tract, kidney, reproductive organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, esophagus, thyroid, adrenals, urinary

bladder, lymph nodes and skeletal muscle. After fixation, the tissues were processed into paraffin blocks, and 3 μm sections were obtained. All sections were stained with hematoxylin and exosin, and subject to histologic analysis. The size and the number of the follicles in the spleen, lymph nodes and Peyer's patches were increased significantly in the AGP-3 transgenic mice (Figure 5, 6 and 7). The spleen, lymph node and Peyer's patches of both the transgenic and the control mice were subject to immunohistology analysis with B cell and T cell specific antibodies. The formalin fixed paraffin embedded sections were deparaffinized and hydrated to deionized water. The sections were quenched with 3% hydrogen peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat monoclonal anti-mouse B220 and CD3 (Harlan, Indianapolis, IN), respectively. The binding was detected by biotinylated rabbit anti-rat immunoglobulins and peroxidase conjugated streptavidin (BioGenex, San Ramon, CA) with DAB as chromagen (BioTek, Santa Barbara, CA). Sections were counterstained with hematoxylin. The B cell numbers, as indicated by positive B220 staining, increased significantly in the spleen, lymph nodes and Peyer's batches (Figure 5, 6, and 7). The T cell numbers, as indicated by the anti-CD3 staining, were slightly decreased. There were no differences in the morphology of the thymus between the transgenic and the control group. By immunohistology, the T cell population was similar in numbers. At 8 weeks of age, there are no distinctive morphologic changes in the liver, kidneys, or urinary, central nervous, hematopoietic, skeletal, respiratory, gastrointestinal, endocrine, or reproductive systems.

After necropsy, MLN and sections of spleen and thymus from 10 AGP-3 transgenic mice (animals 3, 6, 9, 10, 13, 38, 40, 58, 59 and 62) and 5 control littermates (animals 7, 8, 11, 12, and 14) were removed. Single cell suspensions were prepared by gently grinding the tissues with the flat end

of a syringe against the bottom of a 100 µm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells were washed twice in a 15 ml volume then counted. Approximately 1 million cells from each tissue was stained with 0.5 µg antibody in a 100 µl volume of PBS (without Calcium and Magnesium) + 0.1% Bovine Albumin + 0.01% Sodium Azide. All spleen and MLN samples were incubated with 0.5 µg CD16/32(FcγIII/II) Fc block in a 20 µl volume for 10 minutes prior to the addition of FITC or PE-conjugated monoclonal antibodies against CD90.2 (Thy-1.2), CD45R (B220), CD11b(Mac-1), Gr-1, CD4, or CD8 (PharMingen, San Diego, CA) at 2-8 °C for 30 min. The cells were washed then analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). Thymus samples were stained with FITC conjugated anti-Thy-1.2, FITC conjugated anti-CD4, and PE conjugated anti-CD8 (PharMingen, San Diego, CA).

In the MLN of the AGP-3 transgenic mice, the percentage of B220 positive B cells increased by 100% (Figure 6). The percentage of the Thy-1.2 positive T cells decreased approximately 36%, with similar reductions in both CD4(+) and CD8(+) populations. The helper CD4(+) / suppressor CD8(+) ratio remained unchanged. Similar increases in B cell and reductions in T cell populations were also observed in the spleens of the AGP-3 transgenic mice (Figure 8), though to a lesser extent. No obvious changes in staining with anti-CD11b or anti-Gr-1 antibodies were observed in the lymph node and spleen between the transgenic and the control group. In the thymus, there were no differences in the percentages of Thy-1.2(+), CD4(+), CD8(+) or CD4(+)CD8(+) populations between the AGP-3 transgenic and the control mice.

Serum Immunoglobulin and Autoantibody Analysis

Transgenic mice and control littermates were bled successively at 6, 7, 8, 9, 11, and 12 weeks of age. Serum immunoglobulin levels were

quantitated using by ELISA with Mouse Hybridoma Subtype Kit as suggested by manufacture (Boehringer Mannheim, Indianapolis, IN). Presence of autoantibodies directed against nuclear antigens and dsDNA were examined in the serum by enzyme linked immunosorbant assay (ELISA). The levels of anti-nuclear antibodies were detected using ANA screen kit (Sigma) and anti-mouse IgG peroxidase secondary antibody. Mouse serum samples were diluted 1:200 in ANA screen ELISA. For the detection of anti-dsDNA autoantibodies in serum, high binding ELISA plates were coated with plasmid DNA (Immunovision) as an antigen in the presence of methylated BSA. After blocking the non-specific sites and washing, diluted mouse serum samples were added to wells in duplicated and the binding was quantitated using horse radish peroxidase-labeled anti-mouse IgG or anti-mouse IgM reagents (Southern Biotech). A pooled positive serum from BWF1 mice and pooled negative serum from B6 mice was used as controls. Experiment for the detection of anti-histone antibodies was essentially done similar to anti-DNA ELISA except that carbonate-bicarbonate buffer (pH9.6) buffer was used as coating buffer. Serum antibody data were compared by Mann Whitney test using Sigmastat software (SPSS Science, Chicago, IL).

B Cell Survival and Proliferation Assay

Cells were isolated from spleens of 2-4 months old mice by negative selection. Briefly, B lymphocytes were purified by density gradient centrifugation and then passed over a B cells column (Accurate/ Cedarlane, Westbury, NY). Cells isolated by this method were analyzed by flow cytometry and >90% were found positive for B220 staining. Isolated B cells were cultured in MEM+10% FCS at 37°C, 5%CO_2. Cells were collected from triplicate wells daily on day 1 through day 9 and incubated with 5 μg/ml Propidium Iodide. Cells were analyzed by Flow cytometry and the percentage of dead cells was calculated. For B cell

proliferation assay, purified (10^5) B cells from B6 mice as described above were cultured in MEM+10% heat inactivated FCS in triplicate in 96 well flat bottomed plate with/without 2 μg/ml of Goat $F(ab')_2$ anti-mouse IgM (Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania) and/or indicated amount of recombinant AGP-3 for a period of 4 days at 37 °C, 5%CO_2. Proliferation was measured by an uptake of radioactive ^{3}(H) thymidine in last 18 hours of pulse. Data is shown in figure 14 as mean±standard deviation of triplicate wells.

-3; 260-3.

B Cell Hyperplasia and Hypergammaglobulinemia in AGP-3 Transgenic Mice

To gain insights into the biological function for AGP-3, transgenic mice were generated that expressed full-length murine AGP-3 protein driven by the ubiquitous 3-actin promoter. Founder mice harboring the AGP-3 transgene were identified by PCR analysis of genomic DNA samples. Transgene expression was confirmed by RT-PCR from spleen total RNA. At 8 weeks of age, ten AGP-3 transgenic mice and five control littermates were subject to necropsy and pathological analysis. The transgenic mice were of normal size and weight. However, the spleen weight relative to the body weight increased by approximately 45% in the AGP-3 transgenic group as compared to the control mice. The sizes of lymph nodes and Payer's Patches were also increased substantially in all the AGP-3 transgenic mice. Histology analysis demonstrated that the size and the number of the follicles in the spleen, lymph nodes and Payer's patches were increased significantly in the AGP-3 transgenic group (Figure 10). Immunohistology staining with B and T cell specific markers indicated the B cell numbers increased significantly in the spleen, lymph nodes and Payer's patches of the transgenic group (Figure 10). The T cell numbers, as indicated by the anti-CD3 staining, were decreased

correspondingly (Figure 10). There were no differences in the morphology and immunostaining of thymus between the transgenic and the control groups. No changes were observed in other organs or organ systems of the 8 weeks old transgenic mice, including kidney, liver, and hematopoietic tissues.

The B cell hyperplasia phenotype in the AGP-3 transgenic mice was also confirmed by flow cytometry analysis. In the mesenteric lymph nodes of the AGP-3 transgenic mice, the percentage of B220 positive B cells increased by 100% (Figure 11). The percentage of the Thy-1.2 positive T cells decreased by approximately 36%, with similar reductions in both CD4(+) and CD8(+) T cells. Similar increase in B cell and reduction in T cell populations were also observed in the spleens of the AGP-3 transgenic mice, though to a lesser extent (Figure 11). Of note, the total T cell numbers in the lymph node and spleen of AGP-3 transgenic mice were similar to the control littermates. In the thymus, there were no differences in the percentages of single positive CD4(+) or CD8(+) T cells, or CD4(+)CD8(+) populations between the AGP-3 transgenic and the control mice (Figure 11). No obvious changes in staining with anti-CD11b or anti-Gr-1 antibodies were observed in the lymph nodes and spleen between the transgenic and the control group (Figure 11). The histological and FACS analysis, together, suggested severe B cell hyperplasia phenotype in the AGP-3 transgenic mice.

We also examined B cell populations of different developmental stages by FACS analysis. No differences were observed in the percentage of the pro B (B220+IgM-), immature B (B220+IgM+), or mature B (IgM+IgD+) within spleenic B cell population of the AGP-3 transgenic mice as compared to the control littermates. In addition, the number of the spleenic CD5+ B cells in the AGP-3 transgenic mice from 1 to 9 month of age was unaltered. We also didn't detect any alteration of the CD40

expression level on B cells in the transgenic mice, suggesting that the B cell hyperplasia in the AGP-3 transgenic mice was not caused by CD40 upregulation.

In addition to the B cell hyperplasia phenotype, the AGP-3 transgenic mice also had severe hypergammaglobulinemia . The serum globulin level in AGP-3 transgenic mice increased more that 100% as compared to the control group. Total protein level also increased correspondingly in the transgenic, while albumin level remained the same. The increased B cell numbers and high serum globulin level suggested elevated serum immunoglobulin titer. Thus we examined serum levels of IgM, IgG, IgA and IgE of AGP-3 transgenic mice from 6 to 12 weeks of age. Comparing to the same age control littermates, serum IgM, IgG, IgA and IgE were significantly increased in all age groups of AGP-3 transgenic mice. The increase found in serum IgG was not specific to any particular subclass (IgG1, IgG2a, IgG2b, and IgG3). No significant differences in other serum chemistry or hematology parameters were observed at this age. The increased serum immunoglobulin levels is likely to result directly from increased B cell number, but may also be aggravated by increased B cell antibody production.

Autoantibodies associated with lupus in AGP-3 transgenic mice

Increased humoral immunity in AGP-3 transgenic mice warranted us to look for possible phenotypes resembling B cell associated autoimmune diseases such as systemic lupus erythematosus (SLE). The common denominator in lupus patients and lupus prone mice is IgG autoantibody production, and the hallmark of this disease is the presence of elevated anti-nuclear antibodies in the serum. The emergence of anti-DNA antibodies represents one final outcome in the different murine lupus models and patients with SLE. When sera from transgenic and non-transgenic mice at various age were examined for the presence of

autoantibodies recognizing nuclear antigens or dsDNA, two different lines of AGP-3 transgenic mice began to show presence of autoantibodies at around 8 weeks of age (Table 1). The amount of anti-nuclear and anti-dsDNA antibody increased with their age in the transgenic animals (Table 1). More interestingly, at 5 and 8 months of age, AGP-3 transgenic mice showed 5-10 higher amount of anti-dsDNA antibodies compared to age matched lupus prone (NZBxNZW)F1 mice. The presence of autoantibodies in the serum of AGP-3 transgenic mice did not discriminate between gender of mice. Both IgG and IgM antibodies to dsDNA were detected in transgenic animals. Presence of such autoantibodies was undetectable in non-transgenic littermates, as expected.

Immune Complex Deposits in the Kidney of AGP-3 Transgenic Mice

Presence of anti-DNA antibodies followed by immune complex induced renal damage is classical picture seen in lupus associated nephritis. At 5 month of age, the AGP-3 transgenic mice developed glomerular proteinaceous deposits in the kidney (Figure 13). The deposits were seen in more than 60% of the glomeruli in the transgenic mice, but absent in the control littermates. Immunohistology showed the deposits contained moderate amounts of IgG and larger amounts of IgM (Figure 13). Trichrome staining showed no deposit of connective tissues in the glomeruli at 5 month of age. There is also no evidence of any cellular proliferation or presence of inflammatory cells at this age (Figure 13). Interestingly, the kidney lesions progressed as the transgenic mice grew older. At 8 month of age, there was obvious enlargement of glomeruli in the AGP-3 transgenic mice as compared to the age matched control littermates (Figure 13G). In addition, we also detected extensive connective tissue deposits in the enlarged glomeruli (Figure 13G).

Comparing to the 5 month old mice, the 8 month old transgenic mice had increased IgG level in the glomeruli immune complex deposits (Figure 13I). Majority of the glomeruli in the AGP-3 transgenic mice were affected. We also performed serum and urine chemistry analysis of 5 month old and 8 month old AGP-3 transgenic along with the control littermates. No significant differences were noticed in the 5 month old AGP-3 transgenic mice. However, in the 8 month old mice, we observed increases in serum blood urea nitrogen (BUN) and calcium levels and decrease in serum phosphate level. In addition, the 8 month old AGP-3 mice also had increased protein level in the urine. These changes, together, suggest the onset of renal failure in the 8 month old AGP-3 transgenic mice. In conclusion, the high serum autoantibodies followed by the kidney lesions in theAGP-3 transgenic mice clearly resemble to the pathological progression in the SLE patients and lupus prone mice.

AGP-3 Stimulates B Cell Survival and Proliferation : a Possible Mechanism for Autoimmunity

The B cell hyperplasia phenotype in the AGP-3 transgenic mice might arise from increased B cell survival and/or increased B cell proliferation. We first compared the viability of B cells from AGP-3 transgenic mice with that of the control littermates. B cells were isolated from both transgenic or control mice and incubated in minimal essential medium supplemented with 10% heat inactivated fetal bovine serum. Viability of the B cells was measured by FACS analysis for Propidium Iodide uptake (Figure 14A). By day 3, 30% of B cells isolated from the control mice were dead, whereas only 10% of B cells from AGP-3 transgenic mice were dead. By day 5, 70% of B cells from AGP-3 mice were still viable, whereas only 15% of B cell from control littermates were viable. By day 9, almost 50% of the AGP-3 transgenic B cells still remained viable. Therefore, transgenic expression of AGP-3 prolonged B cell

viability. It remains to be determined if this B cell survival stimuli result directly from AGP-3 action on B cells or through its modulation of the immune system.

Recently Schneider et al (Schneider et al., 1999, and Moore et al., 1999) reported co-stimulation of B cell proliferation by BAFF/BLYS with anti-IgM. We found that AGP-3 alone can also stimulates B cell proliferation in a dose dependent manner with an ED_{50} of approximately 3ng/ml (Figure 14B, upper). A ten fold increase of B cell proliferation was detected by AGP-3 treatment at 10 ng/ml concentration as compared to the untreated cells. In our experiment, anti-IgM alone at 2 μg/ml concentration increased B cell proliferation by 24 fold. Treatment with anti-IgM (2 μg/ml) in combination with various doses of AGP-3 led to dose dependent increase of B cell proliferation, with a maximal 13 fold increase as compared anti-IgM treatment alone and a total of 320 fold increase as compared to the untreated cells. Thus, AGP-3 is a potent B cell stimulatory factor. The increased B cell survival and proliferation may together contribute to the B cell hyperplasia and autoimmune lupus like changes in the AGP-3 transgenic mice.

Table 1: Lupus associated autoantibodies in the serum of AGP-3 transgenic mice.

Autoantibodies	Age (months)	AGP-3 tg (n)	Non-tg littermates (n)	p value
Antinuclear antibodies (IgG)[a]	2-3	7^ (9)	1*(8)	
	5-6	9 (9)	1*(8)	
	8-9	8 (8)	1*(6)	
Anti-dsDNA (IgG)[b]	<2	697±284 (7)	277±67 (7)	NS
	3-4	842±351 (7)	235±49 (7)	<.005
	6-7	2515±428 (5)	970±344 (7)	<.019
	8-10	12293±6767 (11)	1070±602 (12)	<.017
Anti-dsDNA (IgM)[b]	<2	275±33 (7)	46±5 (7)	<.001
	3-4	1684±920 (7)	63±13 (7)	<.003
	6-7	6998±5515 (5)	98±14 (7)	<.001
	8-10	13712±9147 (11)	79±14 (12)	<.001
Anti-Histone (Ig)[b]	<2	741±264 (7)	52±8 (7)	<.001
	3-4	837±436 (7)	53±14 (7)	<.003
	6-7	4220±933 (5)	60±10 (7)	<.001
	8-10	16555±4618 (11)	295±173 (12)	<.001

^ includes two weak positive.

* Weak positive

a: Data is shown as number of ANA positive (mean+2sd of transgene negative littermates) mice using ANA screen kit.

b: Data is represented as mean±SE for each group. Values are shown as Units/ml.

NS: not significant

Bacterial Expression of AGP-3 protein

PCR amplification employing the primer pairs and templates described below are used to generate various forms of human AGP3 proteins. One primer of each pair introduces a TAA stop codon and a unique XhoI or SacII site following the carboxy terminus of the gene. The other primer of each pair introduces a unique NdeI site, a N-terminal methionine, and optimized codons for the amino terminal portion of the gene. PCR and thermocycling is performed using standard recombinant DNA methodology. The PCR products are purified, restriction digested, and inserted into the unique NdeI and XhoI or SacII sites of vector pAMG21 (ATCC accession no. 98113) and transformed into the prototrophic E. coli 393 or 2596. Other commonly used E. coli expression vectors and host cells are also suitable for expression. After transformation, the clones are selected, plasmid DNA is isolated and the sequence of the AGP3 binding protein insert is confirmed.

pAMG21-Human AGP3 protein [128-285]

This construct was engineered to be C-terminal 158 amino acids of human AGP3 and have the following N-terminal and C-terminal residues:

NH_2-MNSRNKR ——GALKLL-COOH.

SEQ ID NO: 35

The template to be used for PCR was human AGP3 in pCDNA3.1(+) vector. Oligonucleotides #1761-31 and #1761-33 were the primer pair to be used for PCR and cloning this gene construct.

1761-31:
5'-ATT TGA TTC TAG AAG GAG GAA TAA CAT ATG AAC AGC CGT AAT AAG CGT GCC GTT CAG GGT -3'
(SEQ ID NO:36)

1761-33:

5'-CCG CGG ATC CTC GAG TTA CAG CAG TTT CAA TGC ACC AAA AAA TGT -3'

(SEQ ID NO:37)

pAMG21-Human FLAG-AGP3 protein [128-285]

This construct was engineered to be C-terminal 158 amino acids of human AGP3 preceded with FLAG epitope. The construct encoded following following N-terminal and C-terminal residues:

NH_2-MDYKDDDDKKLNSRNKR------GALKLL-COOH

(SEQ ID NO: 38)

The template to be used for PCR was human AGP3 in pCDNA3.1(+) vector. Oligonucleotides #1761-32 and #1761-33 were the primer pair to be used for PCR and cloning this gene construct.

1761-32:

5'-GAC GAT GAC AAG AAG CTT AAC AGC CGT AAT AAG CGT GCC GTT CAG GGT -3'

(SEQ ID NO:39)

1761-33:

5'-CCG CGG ATC CTC GAG TTA CAG CAG TTT CAA TGC ACC AAA AAA TGT -3'

(SEQ ID NO:37)

E. coli were induced during fermentation, the lysate was applied to Q Sepharose FF (Pharmacia, Piscaataway, NJ) equilibrated in 10 mM Mes pH 6.0 and eluted with 50- 400 mM NaCl gradient over 30 column volumes. Fractions containing AGP-3 were pooled and loaded onto a Q Sepharose HP column (Pharmacia, Piscataway, NJ) equilibrated in 10 mM Tris-HCL pH 8.5. AGP-3 was eluted with an increasing linear NaCl gradient (50 mM-200 mM) over 30 column volumes. Endotoxin was removed by application to Sp HiTRAP column (Pharmacia, Piscataway, NJ) pH 4.8 and eluted with 100-500 mM NaCl in 10 mM sodium acetate pH 4.8 over 25 column volumes. Final endotoxin level of the purified

protein is approximately 0.2 EU/mg. The purified human AGP-3 is truncated at residue Arg133 as indicated by N-terminal sequencing and has a molecular weight of 16.5 KDa by reducing SDS-PAGE. The purified human FLAG-AGP-3 protein is confirmed by N-terminal sequence analysis of the protein. The FLAG-AGP3 protein is recognized by M2 monoclonal antibody against FLAG epitope (Kodak, New Haven, CT).

For europium labeling of the protein, human AGP-3 (lot# 092299) was dialyzed into 50 mM sodium carbonate pH 9. Europium labeling reagent (Wallac Delfia reagent lot# 704394) was dissolved in the same buffer. AGP-3 protein was mixed with a 20-fold molar excess of labeling reagent for 24 hours at room temperature. The mixture was then placed on a Sephadex G-25 column which had been equilibrated in 50 mM Tris-HCl pH 7.8, 150 mM NaCl. The protein was eluted from the column with the same buffer. Protein concentration was determined using the BCA method (Pierce Chemical Co.).

Abbreviations

Abbreviations as used throughout this specification are defined as follows, unless otherwise defined in specific instances.

CDR	complementarity determining region
dsDNA	double-stranded DNA
EST	expressed sequence tag
ORF	open reading frame
SDS	sodium dodecyl sulfate
TNF	tumor necrosis factor

* * *

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the

appended claims cover all such equivalent variations that come within the scope of the invention as claimed.

What is claimed is:

1. An isolated or recombinant polypeptide having a sequence comprising SEQ ID NO: 25, wherein said polypeptide does not comprise SEQ ID NOS: 2, 4, or 5 or a sequence with 90% identity thereto.
2. The polypeptide of Claim 1, further comprising SEQ ID NO: 26.
3. The polypeptide of Claim 1 having a sequence comprising SEQ ID NO: 27.
4. The polypeptide of Claim 1, wherein said polypeptide does not comprise SEQ ID NOS: 2, 4, or 5 or a sequence with 80% identity to SEQ ID NOS: 2, 4, or 5.
5. The polypeptide of Claim 1, comprising an Fc-region.
6. The polypeptide of Claim 1, wherein the polypeptide has the structure

$$(X^1)_a\text{-}F^1\text{-}(X^2)_b$$

wherein:

F^1 is a vehicle;

X^1 and X^2 are each independently selected from $-(L^1)_c\text{-}P^1$, $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2$, $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3$, and $-(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2\text{-}(L^3)_e\text{-}P^3\text{-}(L^4)_f\text{-}P^4$

P^1, P^2, P^3, and P^4 are each independently selected from SEQ ID NOS: 6, 25, 26, and 27;

L^1, L^2, L^3, and L^4 are each independently linkers; and

a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1.

7. The composition of matter of Claim 6 of the formula

$$X^1\text{-}F^1$$

or

$$F^1\text{-}X^2.$$

8. The composition of matter of Claim 6 of the formula

$$F^1\text{-}(L^1)_c\text{-}P^1.$$

9. The composition of matter of Claim 6 of the formula

$$F^1\text{-}(L^1)_c\text{-}P^1\text{-}(L^2)_d\text{-}P^2.$$

10. The composition of matter of Claim 6 wherein F^1 is an IgG Fc domain.

11. The composition of matter of Claim 6 wherein F^1 is an IgG1 Fc domain.

12. The polypeptide of Claim 1, wherein the polypeptide comprises an antibody sequence in which one or more amino acids from antibody CDR regions are replaced by sequences selected from SEQ ID NOS: 6, 25, 26, and 27.

13. The polypeptide of Claim 12, wherein a first CDR region is replaced by SEQ ID NO: 25 and a second CDR region is replaced by SEQ ID NO: 26.

14. The polypeptide of Claim 1, wherein the polypeptide comprises a sequence selected from SEQ ID NOS: 7 TO 24 and 40 shown in Figure 9, except that the B/B' region is replaced by SEQ ID NO: 25.

15. The polypeptide of Claim 1, wherein the polypeptide comprises a sequence selected from SEQ ID NOS: 7 TO 24 and 40 shown in Figure 9, except that the B/B' region is replaced by SEQ ID NO: 25 and the E/F region is replaced by SEQ ID NO: 26.

16. The polypeptide of Claim 1, wherein the polypeptide comprises a sequence selected from SEQ ID NOS: 7 TO 24 and 40 shown in Figure 9, except that the B/I region is replaced by SEQ ID NO: 27.

17. A polypeptide of Claim 1 capable of eliciting B cell growth, survival, or activation in mesenteric lymph nodes.

18. The protein of any of Claims 1, 2, 3, 4, 14, 15, 16, or 17, wherein the protein is covalently linked to a water-soluble polymer or a carbohydrate.

19. The protein of Claim 18, wherein the polymer is polyethylene glycol.

20. The protein of Claim 18, wherein the carbohydrate is dextran.

21. An isolated nucleic acid encoding a protein of any of Claims 1 to 17.

22. The nucleic acid of Claim 21 including one or more codons preferred for Escherichia coli expression.

23. The nucleic acid of Claim 21 having a detectable label attached thereto.

24. An expression vector comprising the nucleic acid of Claim 21.

25. A host cell transformed or transfected with the expression vector of Claim 24.

26. The host cell of Claim 25, wherein the cell is a prokaryotic cell.

27. The host cell of Claim 26, wherein the cell is Escherichia coli.

28. A method to assess the ability of a candidate compound to bind to an AGP-3 related protein comprising:

(a) incubating a polypeptide of Claim 1 with the candidate compound under conditions that allow binding; and

(b) measuring the bound compound.

29. A method of regulating expression of an AGP-3 related protein in an animal comprising administering to the animal a nucleic acid complementary to the nucleic acid of Claim 22.

30. A pharmaceutical composition comprising a therapeutically effective amount of a protein of Claim 1 in a pharmaceutically acceptable carrier, adjuvant, solubilizer, stabilizer and/or anti-oxidant.

31. A method of modulating B cell growth, survival, or activation in a mammal, which comprises administering a therapeutically effective amount of a modulator of an AGP-3 related protein.

32. The method of Claim 31, wherein the B cell growth, survival, or activation takes place in mesenteric lymph nodes.

33. An antibody that specifically binds to SEQ ID NOS: 25, 26, or 27.

34. The antibody of claim 33, wherein the antibody is a monoclonal antibody.

35. The antibody of claim 33, wherein the antibody was generated by phage display.

36. A method of modulating B cell growth, survival, or activation in a mammal comprising administering a therapeutically effective amount of the antibody of Claim 33.

37. The method of Claim 36, wherein the B cell growth, survival, or activation takes place in mesenteric lymph nodes.

38. A method to modulate hybridoma cell antibody production, which comprises treating hybridoma cells with the compound of Claim 1.

FIG. 1A

```
        10                  30                  50
GAATTCGGCACGAGCTGAGGGGTGAGCCAAGCCCTGCCATGTAGTGCACGCAGGACATCA
        70                  90                  110
ACAAACACAGATAACAGGAAATGATCCATTCCCTGTGGTCACTTATTCTAAAGGCCCCAA
        130                 150                 170
CCTTCAAAGTTCAAGTAGTGATATGGATGACTCCACAGAAAGGGAGCAGTCACGCCTTAC
                        M   D   D   S   T   E   R   E   Q   S   R   L   T
        190                 210                 230
TTCTTGCCTTAAGAAAAGAGAAGAAATGAAACTGAAGGAGTGTGTTTCCATCCTCCCACG
 S   C   L   K   K   R   E   E   M   K   L   K   E   C   V   S   I   L   P   R
        250                 270                 290
GAAGGAAAGCCCCTCTGTCCGATCCTCCAAAGACGGAAAGCTGCTGGCTGCAACCTTGCT
 K   E   S   P   S   V   R   S   S   K   D   G   K   L   L   A   A   T   L   L
        310                 330                 350
GCTGGCACTGCTGTCTTGCTGCCTCACGGTGGTGTCTTTCTACCAGGTGGCCGCCCTGCA
 L   A   L   L   S   C   C   L   T   V   V   S   F   Y   Q   V   A   A   L   Q
        370                 390                 410
AGGGGACCTGGCCAGCCTCCGGGCAGAGCTGCAGGGCCACCACGCGGAGAAGCTGCCAGC
 G   D   L   A   S   L   R   A   E   L   Q   G   H   H   A   E   K   L   P   A
        430                 450                 470
AGGAGCAGGAGCCCCCAAGGCCGGCCTGGAGGAAGCTCCAGCTGTCACCGCGGGACTGAA
 G   A   G   A   P   K   A   G   L   E   E   A   P   A   V   T   A   G   L   K
        490                 510                 530
AATCTTTGAACCACCAGCTCCAGGAGAAGGCAACTCCAGTCAGAACAGCAGAAATAAGCG
 I   F   E   P   P   A   P   G   E   G   N   S   S   Q   N   S   R   N   K   R
        550                 570                 590
TGCCGTTCAGGGTCCAGAAGAAACAGTCACTCAAGACTGCTTGCAACTGATTGCAGACAG
 A   V   Q   G   P   E   E   T   V   T   Q   D   C   L   Q   L   I   A   D   S
```

FIG. 1B

```
      610                 630                 650
TGAAACACCAACTATACAAAAAGGATCTTACACATTTGTTCCATGGCTTCTCAGCTTTAA
 E  T  P  T  I  Q  K  G  S  Y  T  F  V  P  W  L  L  S  F  K
      670                 690                 710
AAGGGGAAGTGCCCTAGAAGAAAAAGAGAATAAAATATTGGTCAAAGAAACTGGTTACTT
 R  G  S  A  L  E  E  K  E  N  K  I  L  V  K  E  T  G  Y  F
      730                 750                 770
TTTTATATATGGTCAGGTTTTATATACTGATAAGACCTACGCCATGGGACATCTAATTCA
 F  I  Y  G  Q  V  L  Y  T  D  K  T  Y  A  M  G  H  L  I  Q
      790                 810                 830
GAGGAAGAAGGTCCATGTCTTTGGGGATGAATTGAGTCTGGTGACTTTGTTTCGATGTAT
 R  K  K  V  H  V  F  G  D  E  L  S  L  V  T  L  F  R  C  I
      850                 870                 890
TCAAAATATGCCTGAAACACTACCCAATAATTCCTGCTATTCAGCTGGCATTGCAAAACT
 Q  N  M  P  E  T  L  P  N  N  S  C  Y  S  A  G  I  A  K  L
      910                 930                 950
GGAAGAAGGAGATGAACTCCAACTTGCAATACCAAGAGAAAATGCACAAATATCACTGGA
 E  E  G  D  E  L  Q  L  A  I  P  R  E  N  A  Q  I  S  L  D
      970                 990                1010
TGGAGATGTCACATTTTTTGGTGCATTGAAACTGCTGTGACCTACTTACACCATGTCTGT
 G  D  V  T  F  F  G  A  L  K  L  L
     1030                1050                1070
AGCTATTTTCCTCCCTTTCTCTGTACCTCTAAGAAGAAAGAATCTAACTGAAAATACCAA
     1090                1110                1130
AAAAAAAAAAAAAAAAAAAAAAAAAAGTAGTTAAAAAAAAAAAAAAAAAAAAAAAAAAA
     1150                1170
AAAAAAAAAAAAAAAAAAAAAACTCGGAGGGGG
```

FIG. 2A

```
        10                  30                  50
GAATTCGGCACGAGCTCCAAAGGCCTAGACCTTCAAAGTGCTCCTCGTGGAATGGATGAG
                                                  M  D  E
        70                  90                  110
TCTGCAAAGACCCTGCCACCACCGTGCCTCTGTTTTTGCTCCGAGAAAGGAGAAGATATG
S  A  K  T  L  P  P  P  C  L  C  F  C  S  E  K  G  E  D  M
      130                 150                 170
AAAGTGGGATATGATCCCATCACTCCGCAGAAGGAGGAGGGTGCCTGGTTTGGGATCTGC
K  V  G  Y  D  P  I  T  P  Q  K  E  E  G  A  W  F  G  I  C
      190                 210                 230
AGGGATGGAAGGCTGCTGGCTGCTACCCTCCTGCTGGCCCTGTTGTCCAGCAGTTTCACA
R  D  G  R  L  L  A  A  T  L  L  L  A  L  L  S  S  S  F  T
      250                 270                 290
GCGATGTCCTTGTACCAGTTGGCTGCCTTGCAAGCAGACCTGATGAACCTGCGCATGGAG
A  M  S  L  Y  Q  L  A  A  L  Q  A  D  L  M  N  L  R  M  E
      310                 330                 350
CTGCAGAGCTACCGAGGTTCAGCAACACCAGCCGCCGCGGGTGCTCCAGAGTTGACCGCT
L  Q  S  Y  R  G  S  A  T  P  A  A  A  G  A  P  E  L  T  A
      370                 390                 410
GGAGTCAAACTCCTGACACCGGCAGCTCCTCGACCCCACAACTCCAGCCGCGGCCACAGG
G  V  K  L  L  T  P  A  A  P  R  P  H  N  S  S  R  G  H  R
      430                 450                 470
AACAGACGCGCTTTCCAGGGACCAGAGGAAACAGAACAAGATGTAGACCTCTCAGCTCCT
N  R  R  A  F  Q  G  P  E  E  T  E  Q  D  V  D  L  S  A  P
      490                 510                 530
CCTGCACCATGCCTGCCTGGATGCCGCCATTCTCAACATGATGATAATGGAATGAACCTC
P  A  P  C  L  P  G  C  R  H  S  Q  H  D  D  N  G  M  N  L
      550                 570                 590
AGAAACATCATTCAAGACTGTCTGCAGCTGATTGCAGACAGCGACACGCCGACTATACGA
R  N  I  I  Q  D  C  L  Q  L  I  A  D  S  D  T  P  T  I  R
```

FIG. 2B

```
      610                 630                 650
AAAGGAACTTACACATTTGTTCCATGGCTTCTCAGCTTTAAAAGAGGAAATGCCTTGGAG
K  G  T  Y  T  F  V  P  W  L  L  S  F  K  R  G  N  A  L  E
      670                 690                 710
GAGAAAGAGAACAAAATAGTGGTGAGGCAAACAGGCTATTTCTTCATCTACAGCCAGGTT
E  K  E  N  K  I  V  V  R  Q  T  G  Y  F  F  I  Y  S  Q  V
      730                 750                 770
CTATACACGGACCCCATCTTTGCTATGGGTCATGTCATCCAGAGGAAGAAAGTACACGTC
L  Y  T  D  P  I  F  A  M  G  H  V  I  Q  R  K  K  V  H  V
      790                 810                 830
TTTGGGGACGAGCTGAGCCTGGTGACCCTGTTCCGATGTATTCAGAATATGCCCAAAACA
F  G  D  E  L  S  L  V  T  L  F  R  C  I  Q  N  M  P  K  T
      850                 870                 890
CTGCCCAACAATTCCTGCTACTCGGCTGGCATCGCGAGGCTGGAAGAAGGAGATGAGATT
L  P  N  N  S  C  Y  S  A  G  I  A  R  L  E  E  G  D  E  I
      910                 930                 950
CAGCTTGCAATTCCTCGGGAGAATGCACAGATTTCACGCAACGGAGACGACACCTTCTTT
Q  L  A  I  P  R  E  N  A  Q  I  S  R  N  G  D  D  T  F  F
      970                 990                1010
GGTGCCCTAAAACTGCTGTAACTCACTTGCTGGAGTGCGTGATCCCCTTCCCTCGTCTTC
G  A  L  K  L  L
     1030                1050                1070
TCTGTACCTCCGAGGGAGAAACAGACGACTGGAAAAACTAAAAGATGGGGAAAGCCGTCA
     1090                1110                1130
GCGAAAGTTTTCTCGTGACCCGTTGAATCTGATCCAAACCAGGAAATATAACAGACAGCC
     1150                1170                1190
```

FIG. 3A

```
      1                                                  50
Hagp3  MDDSTER.EQ SRLTSCLKKR EEMKLKECVS ILPRKESPSV RSSKDGKLLA
Magp3  MDESAKTLPP PCLCFCSEKG EDMKVGYDPI TPQKEEGAWF GICRDGRLLA
 cons  MD.S..... ..L..C..K. E.MK..... .....E.... ....DG.LLA

      51                                                 100
Hagp3  ATLLLALLSC CLTVVSFYQV AALQGDLASL RAELQGHHAE KLPAGAGAPK
Magp3  ATLLLALLSS SFTAMSLYQL AALQADLMNL RMELQSYRGS ATPAAAGAPE
 cons  ATLLLALLS. ..T..S.YQ. AALQ.DL..L R.ELQ..... ..PA.AGAP.

     101                                                 150
Hagp3  AGLEEAPAVT AGLKIFEPPA PGEGNSSQNS RNKRAVQGPE ET........
Magp3  ........LT AGVKLLTPAA PRPHNSSRGH RNRRAFQGPE ETEQDVDLSA
 cons  .........T AG.K...P.A P...NSS... RN.RA.QGPE ET........

      151                          B                  200
Hagp3  .......... .......... ...VTQDCLQ LIADSETPTI QKGSYTFVPW
Magp3  PPAPCLPGCR HSQHDDNGMN LRNIIQDCLQ LIADSDTPTI RKGTYTFVPW
 cons  .......... .......... .....QDCLQ LIADS.TPTI .KG.YTFVPW
```

FIG. 3B

```
          B'        C'        C              D                  E          250
Hagp3   LLSFKRGSAL EEKENKILVK ETGYFFIYGQ VLYTDKTYAM GHLIQRKKVH
Magp3   LLSFKRGNAL EEKENKIVVR QTGYFFIYSQ VLYTDPIFAM GHVIQRKKVH
 cons   LLSFKRG.AL EEKENKI.V. .TGYFFIY.Q VLYTD...AM GH.IQRKKVH

          251     F                        G                H          300
Hagp3   VFGDELSLVT LFRCIQNMPE TLPNNSCYSA GIAKLEEGDE LQLAIPRENA
Magp3   VFGDELSLVT LFRCIQNMPK TLPNNSCYSA GIARLEEGDE IQLAIPRENA
 cons   VFGDELSLVT LFRCIQNMP. TLPNNSCYSA GIA.LEEGDE .QLAIPRENA

          301          I     317
Hagp3   QISLDGDVTF FGALKLL
Magp3   QISRNGDDTF FGALKLL
 cons   QIS..GD.TF FGALKLL
```

FIG. 4A

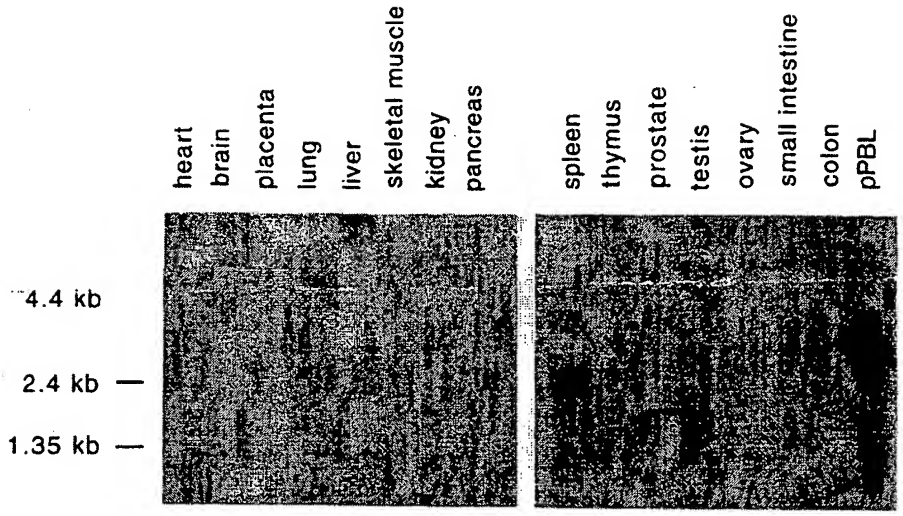

heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
spleen
thymus
prostate
testis
ovary
small intestine
colon
pPBL
4.4 kb
2.4 kb
1.35 kb

FIG. 4B

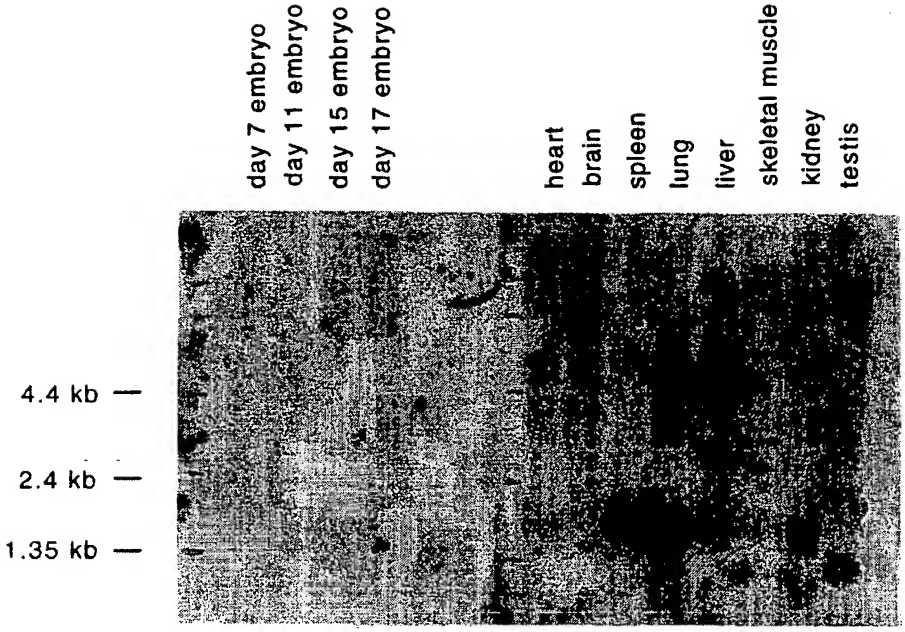

day 7 embryo
day 11 embryo
day 15 embryo
day 17 embryo
heart
brain
spleen
lung
liver
skeletal muscle
kidney
testis
4.4 kb
2.4 kb
1.35 kb

FIG. 5A

FIG. 5B

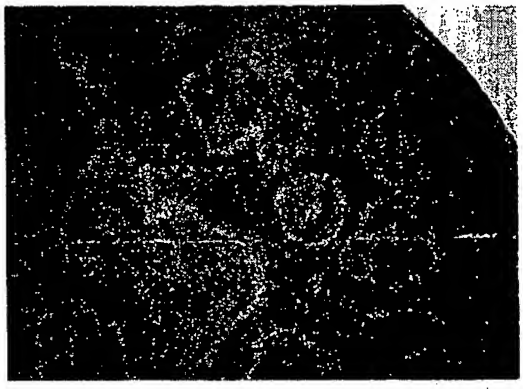


FIG. 5C

FIG. 5D

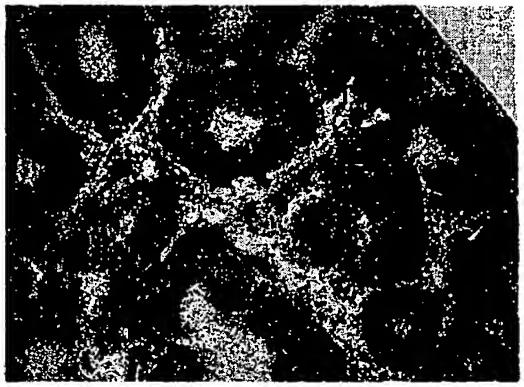


FIG. 5E

FIG. 5F

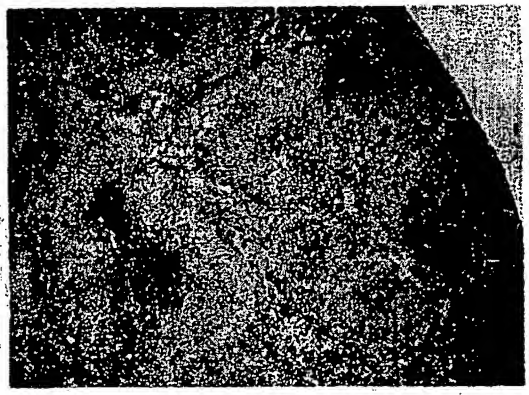


FIG. 6A

FIG. 6B

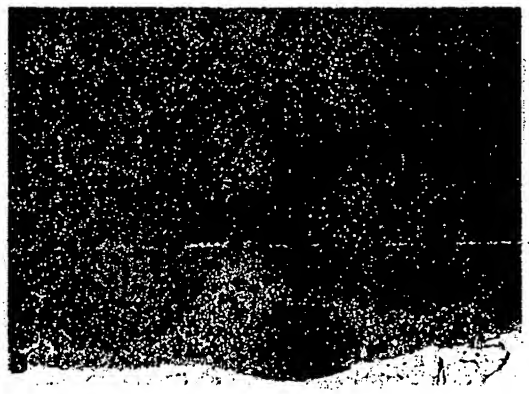


FIG. 6C

FIG. 6D

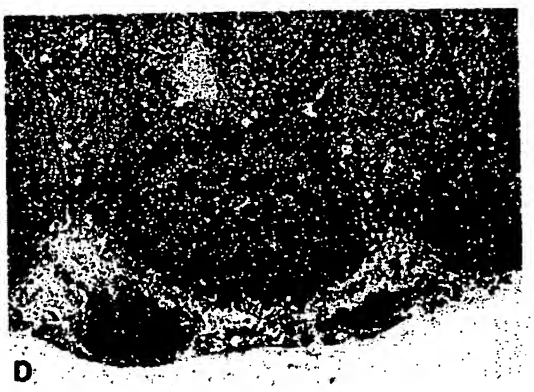


FIG. 6E

FIG. 6F

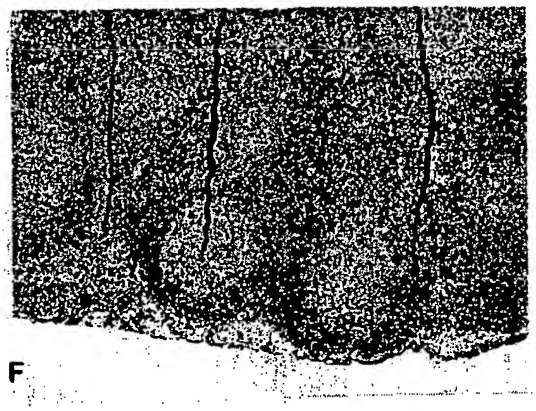


FIG. 7A

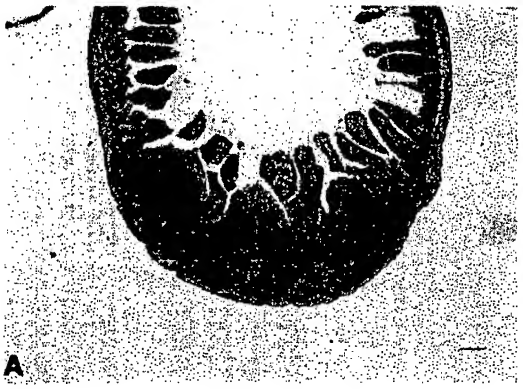


FIG. 7B

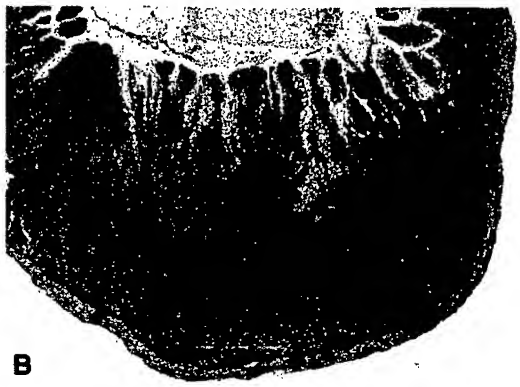


FIG. 7C

FIG. 7D

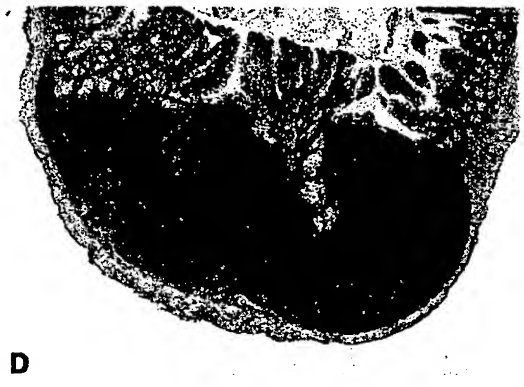


FIG. 7E

FIG. 7F

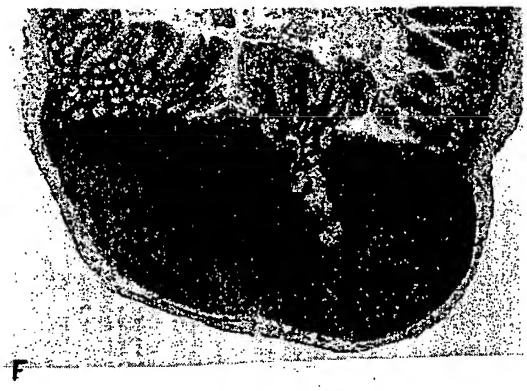


FIG. 8A

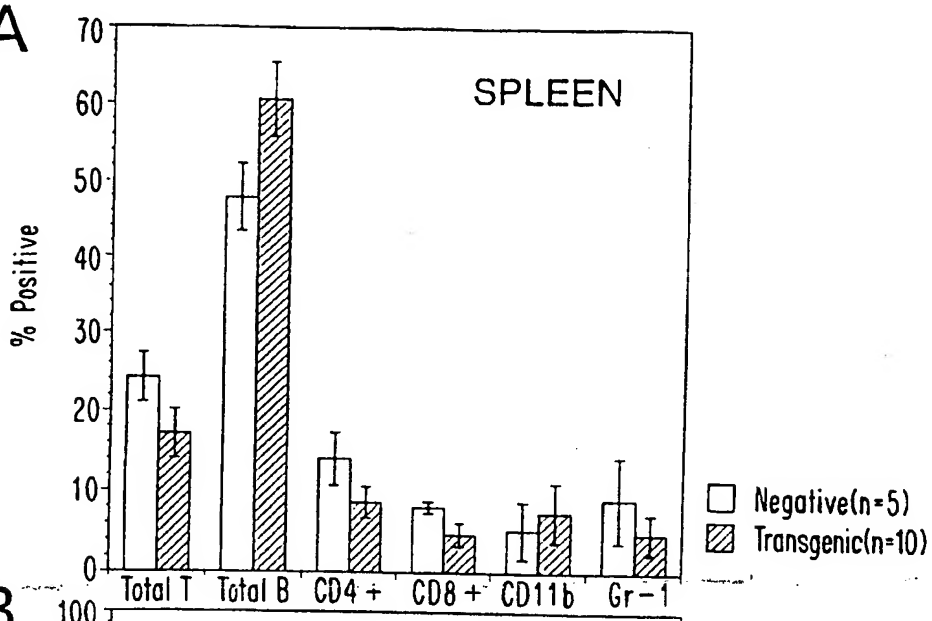


FIG. 8B

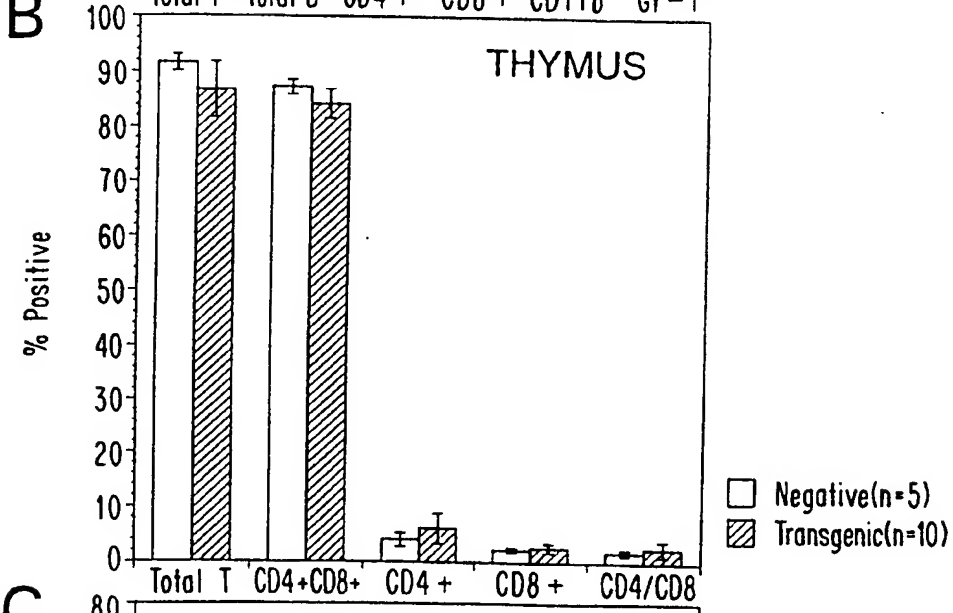


FIG. 8C

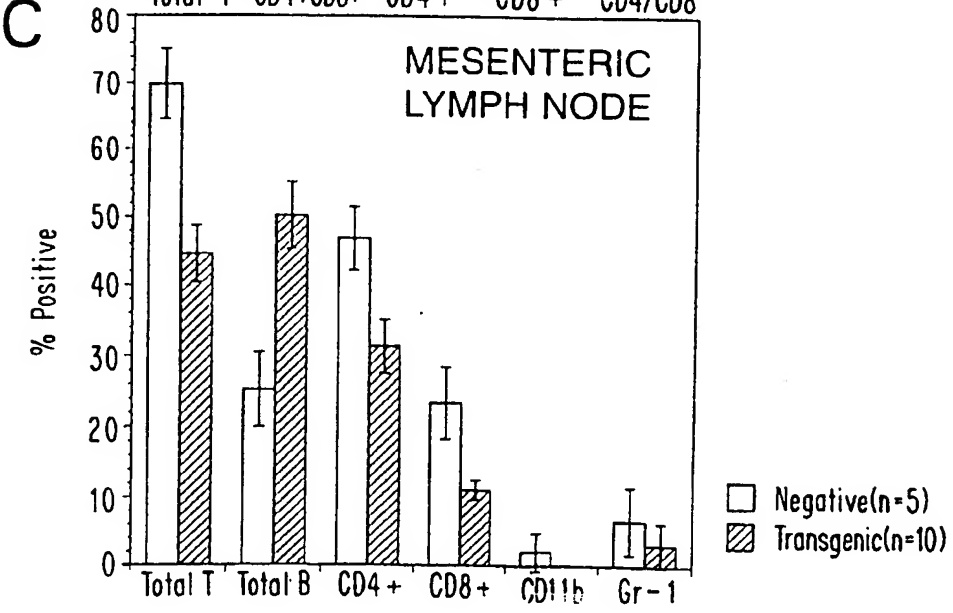
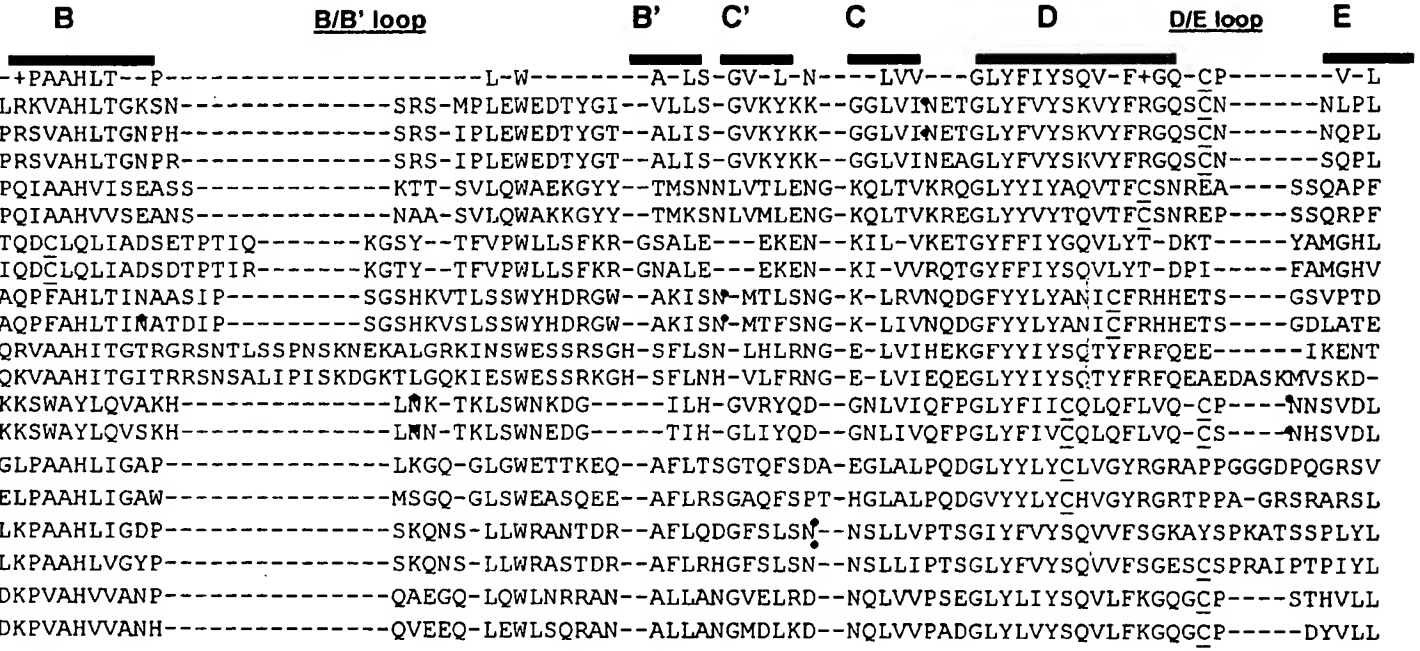
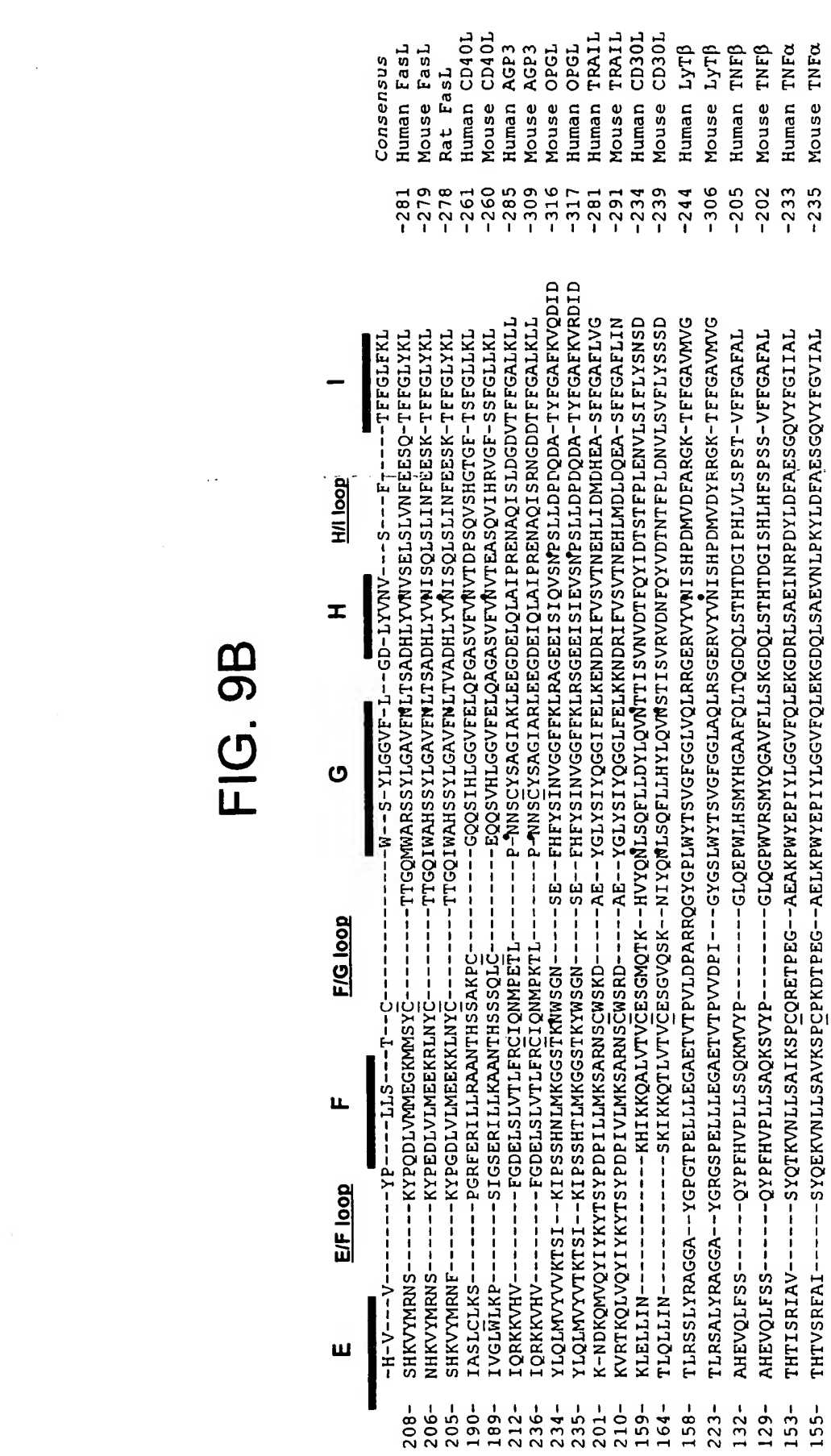


FIG. 9A



SUBSTITUTE SHEET (RULE 26)

FIG. 9B


E
E/F loop
F
F/G loop
G
H
H/I loop
I
-H-V---V--------YP----LLS---T--C----------W--S-YLGGVF-L--GD-LYVNV---S---F-----TFFGLFKL
SHKVYMRNS------KYPQDLVMMEGKMMSYC---------TTGQMWARSSYILGAVFNLTSADHLYVNVSELSLVNFEESQ-TFFGLYKL
NHKVYMRNS------KYPEDLVLMEEKRLNYC---------TTGQIWAHSSYILGAVFNLTSADHLYVNISQLSLINFESK-TFFGLYKL
SHKVYMRNF------KYPGDLVLMEEKKLNYC---------TTGQIWAHSSYILGAVFNLTVADHLYVNISQLSLINFEESK-TFFGLYKL
IASLCLKS--------PGRFERILLRAANTHSSAKPC---------GQQSIHLGGVFELQPGASVFVNVTDPSQVSHGTGF-TSFGLLKL
IVGLWLKP--------SIGSERILLKAANTHSSSQLC---------EQQSVHLGGVFELQAGASVFVNVTEASQVIHRVGF-SSFGLLKL
IQRKKVHV---------FGDELSLVTLFRCIQNMPETL---------P-NNSCYSAGIARLEEGDEIQLAIPRENAQISLDGDVTFFGALKLL
IQRKKVHV---------FGDELSLVTLFRCIQNMPKTL---------P-NNSCYSAGIARLEEGDEIQLAIPRENAQISRNGDDTFFGALKLL
YLQLMVYVVKTSI--KIPSSHNLMKGGSTKTWSGN-----SE--FHFYSINVGGFFKLRAGEEISIQVSNPSLLDPDQDA-TYFGAFKVQDID
YLQLMVYVTKTSI--KIPSSHTLMKGGSTKYWSGN-----SE--FHFYSINVGGFFKLRSGEEISIEVSNPSLLDPDQDA-TYFGAFKVRDID
K-NDKQMVQYIYKYTSYPDDPILLMKSARNSCWSKD-----AE--YGLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEA-SFFGAFLVG
KVRTKQLVQYIYKYTSYPDDPIVLMKSARNSCWSRD-----AE--YGLYSIYQGGLFELKKNDRIFVSVTNEHLMDLDQEA-SFFGAFLIN
KLELLIN----------KHIKKQALVTVCESGMQTK--HVYQNLSQFLLDYLQVNTTISVNVDTFQYIDTSTFPLENVLSIFLYSNSD
TLQLLIN----------SKIKKQTLVTVCESGVQSK--NIYQNLSQFLLHYLQVNSTISVRVDNFQYVDTNTFPLDNVLSVFLYSSSD
TLRSSLYRAGGA--YGPGTPELLLEGAETVTPVLDPARRQGYGPLWYTSVGFGGLVQLRRGERVYVNISHPDMVDFARGK-TFFGAVMVG
TLRSALYRAGGA--YGRGSPELLLEGAETVTPVVDPI---GYGSLWYTSVGFGGLAQLRSGERVYVNISHPDMVDYERGK-TFFGAVMVG
AHEVQLFSS--------QYPFHVPLLSSQKMVYP---------GLQEPWIHSMYHGAAFQLTQGDQLSTHTDGIPHLVLSPST-VFFGAFAL
AHEVQLFSS--------QYPFHVPLLSAQKSVYP---------GLQGPWVRSMYQGAVFLLSKGDQLSTHTDGISHLHFSPSS-VFFGAFAL
THTISRIAV--------SYQTKVNLLSAIKSPCQRETPEG--AEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL
THTVSRFAI--------SYQEKVNLLSAVKSPCPKDTPEG--AELKPWYEPIYLGGVFQLEKGDQLSAEVNLPKYLDFAESGQVYFGVIAL
Consensus
-281 Human FasL
-279 Mouse FasL
-278 Rat FasL
-261 Human CD40L
-260 Mouse CD40L
-285 Human AGP3
-309 Mouse AGP3
-316 Mouse OPGL
-317 Human OPGL
-281 Human TRAIL
-291 Mouse TRAIL
-234 Human CD30L
-239 Mouse CD30L
-244 Human LyTβ
-306 Mouse LyTβ
-205 Human TNFβ
-202 Mouse TNFβ
-233 Human TNFα
-235 Mouse TNFα

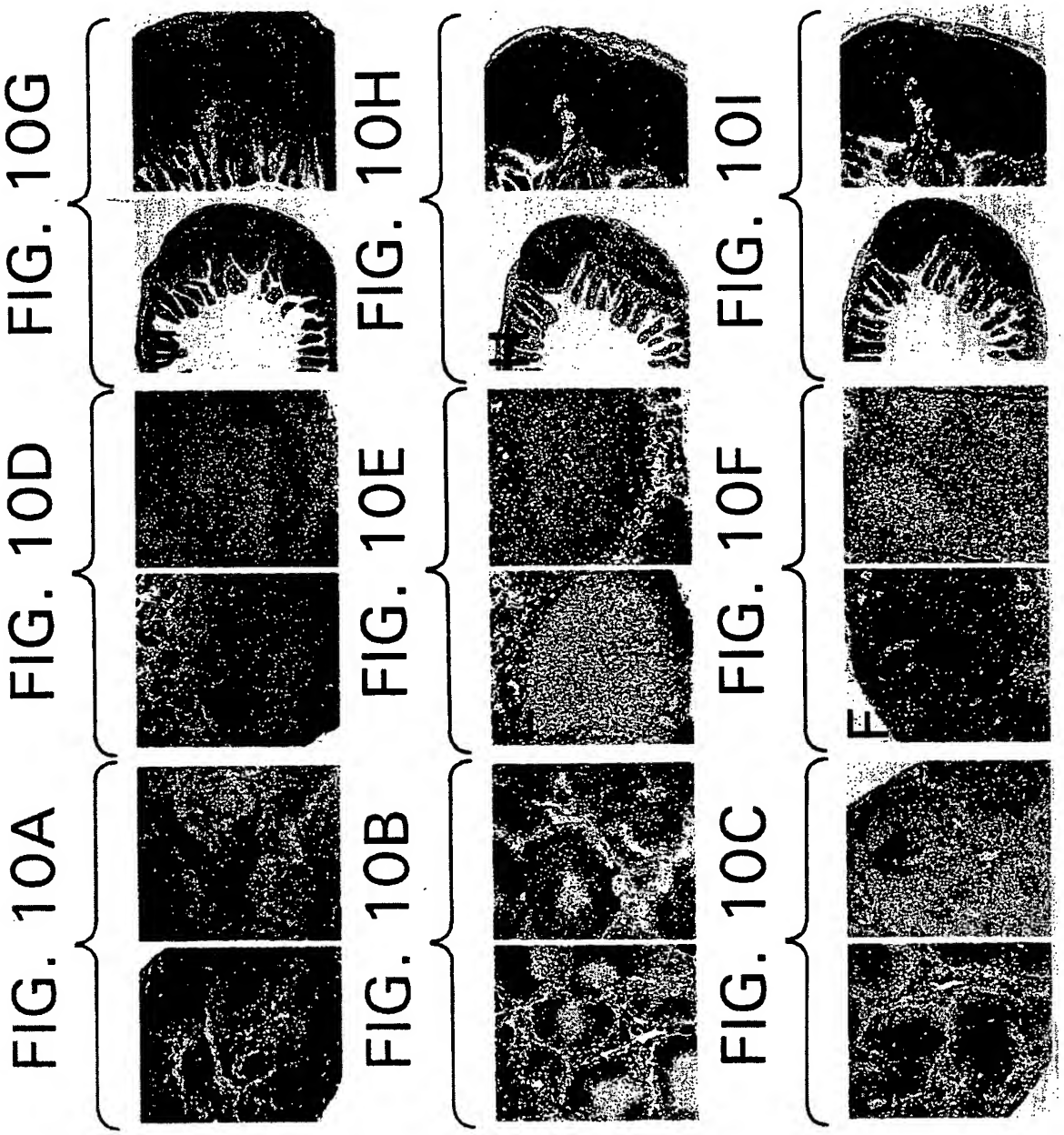


FIG. 10A
FIG. 10B
FIG. 10C
FIG. 10D
FIG. 10E
FIG. 10F
FIG. 10G
FIG. 10H
FIG. 10I

FIG. 11A

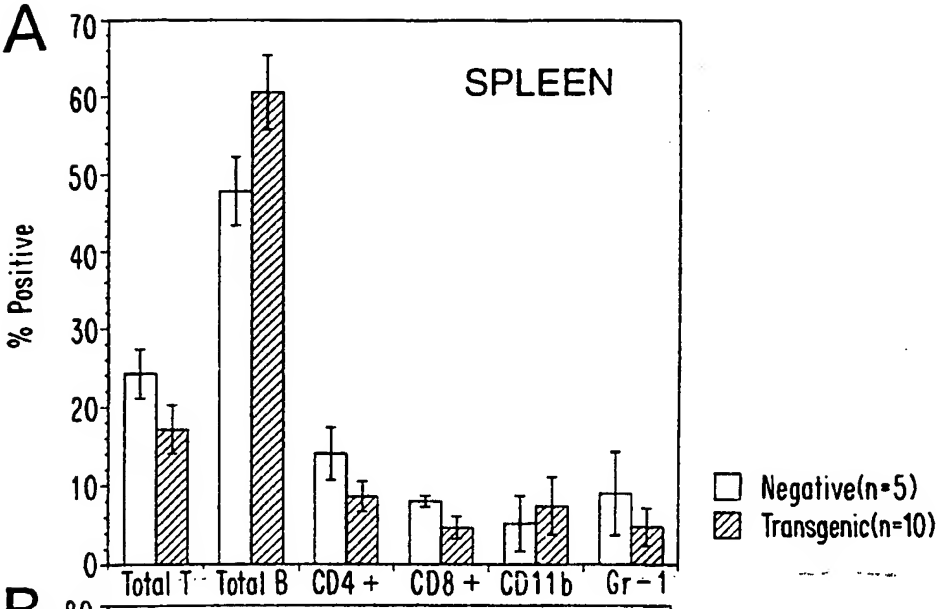


FIG. 11B

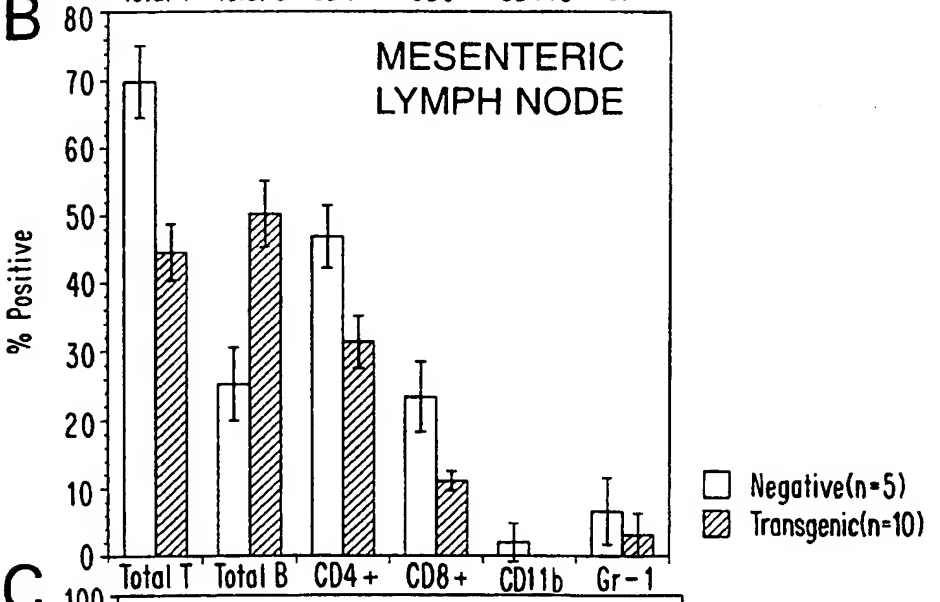


FIG. 11C

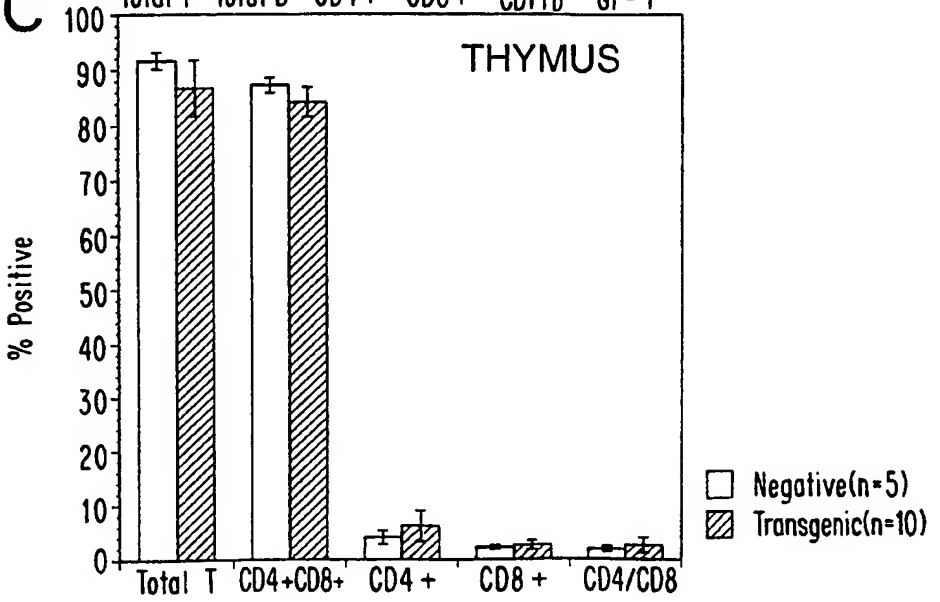


FIG. 12A

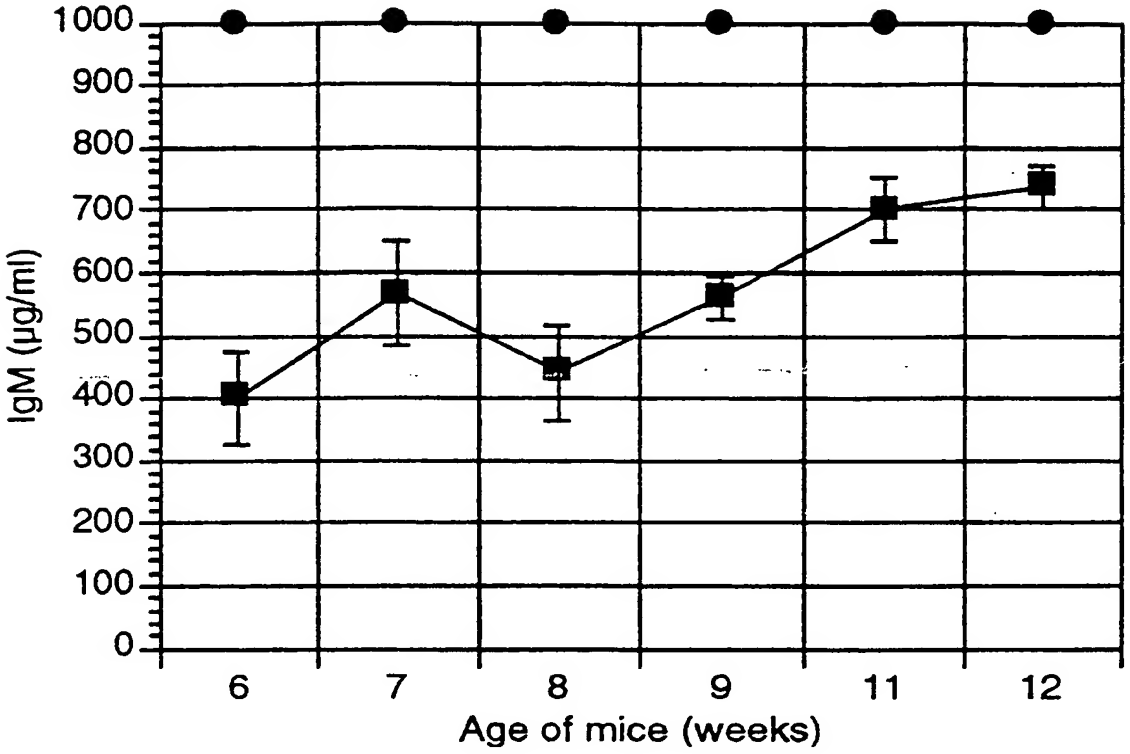


FIG. 12B

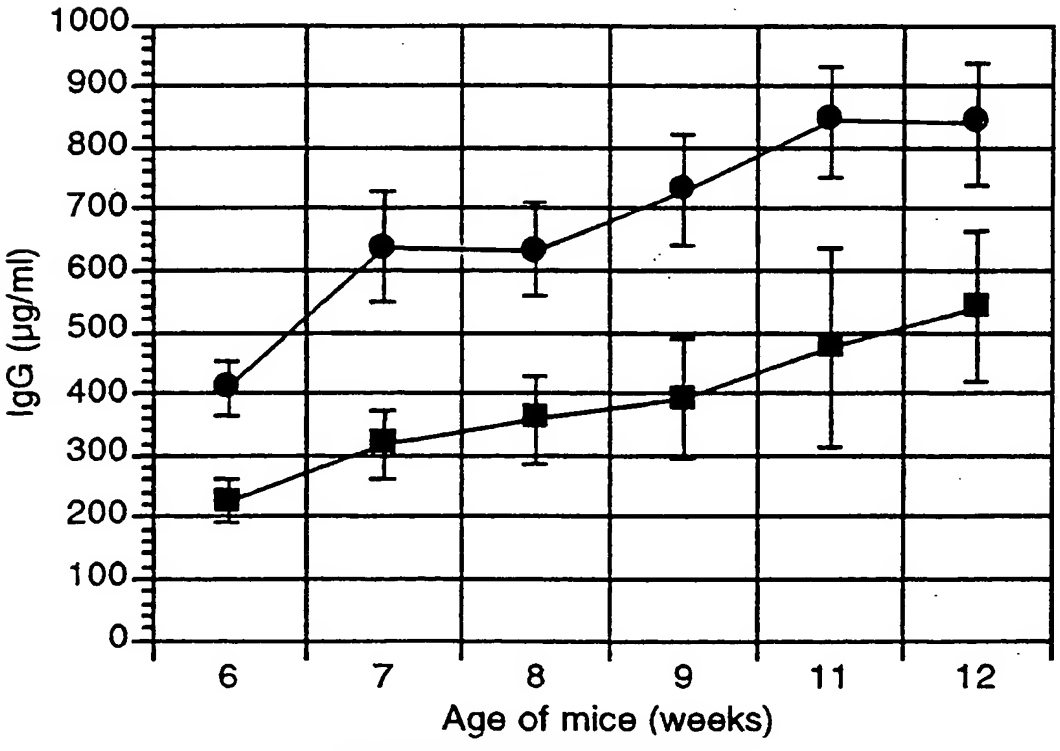


FIG. 12C

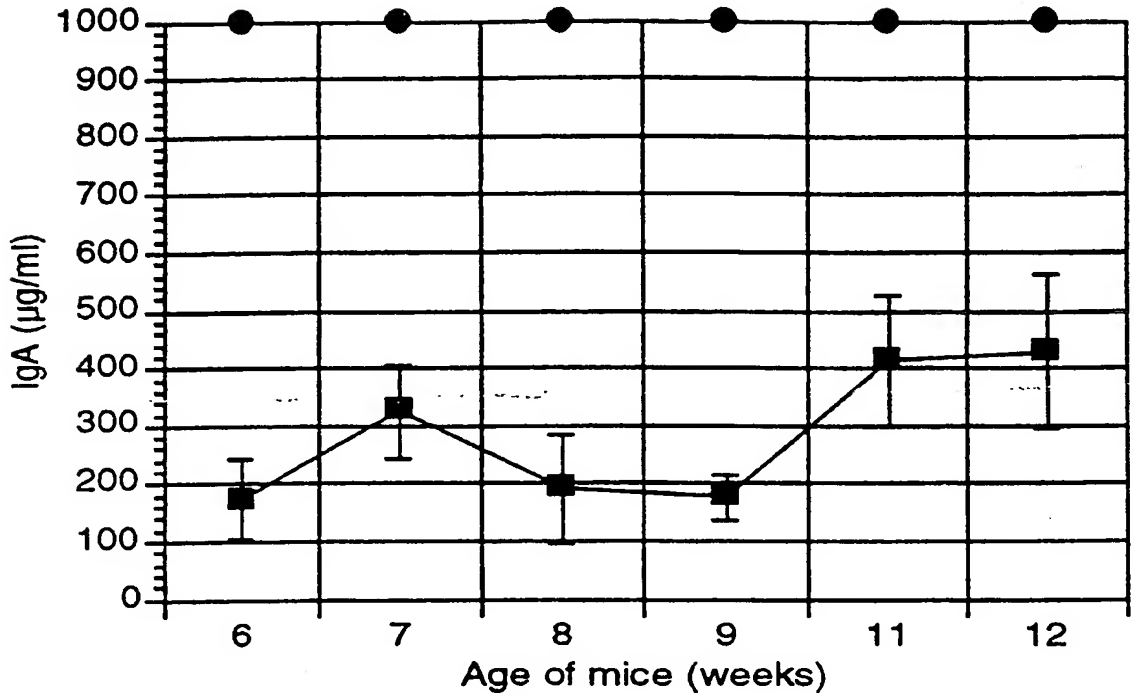
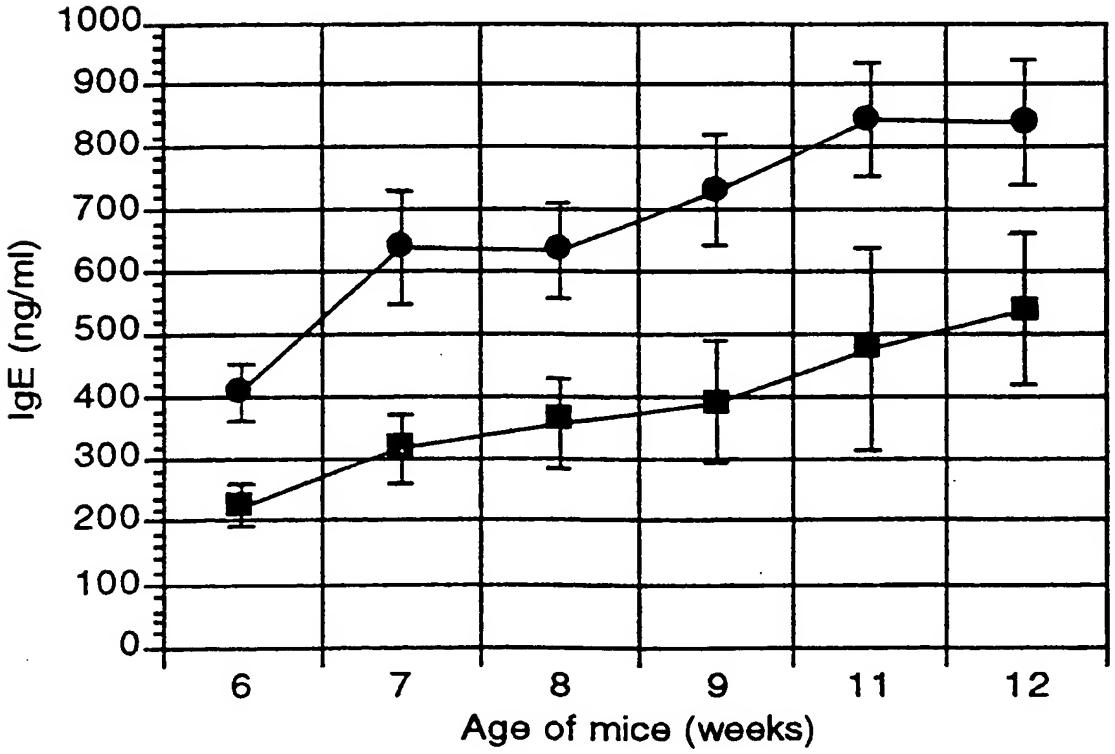


FIG. 12D



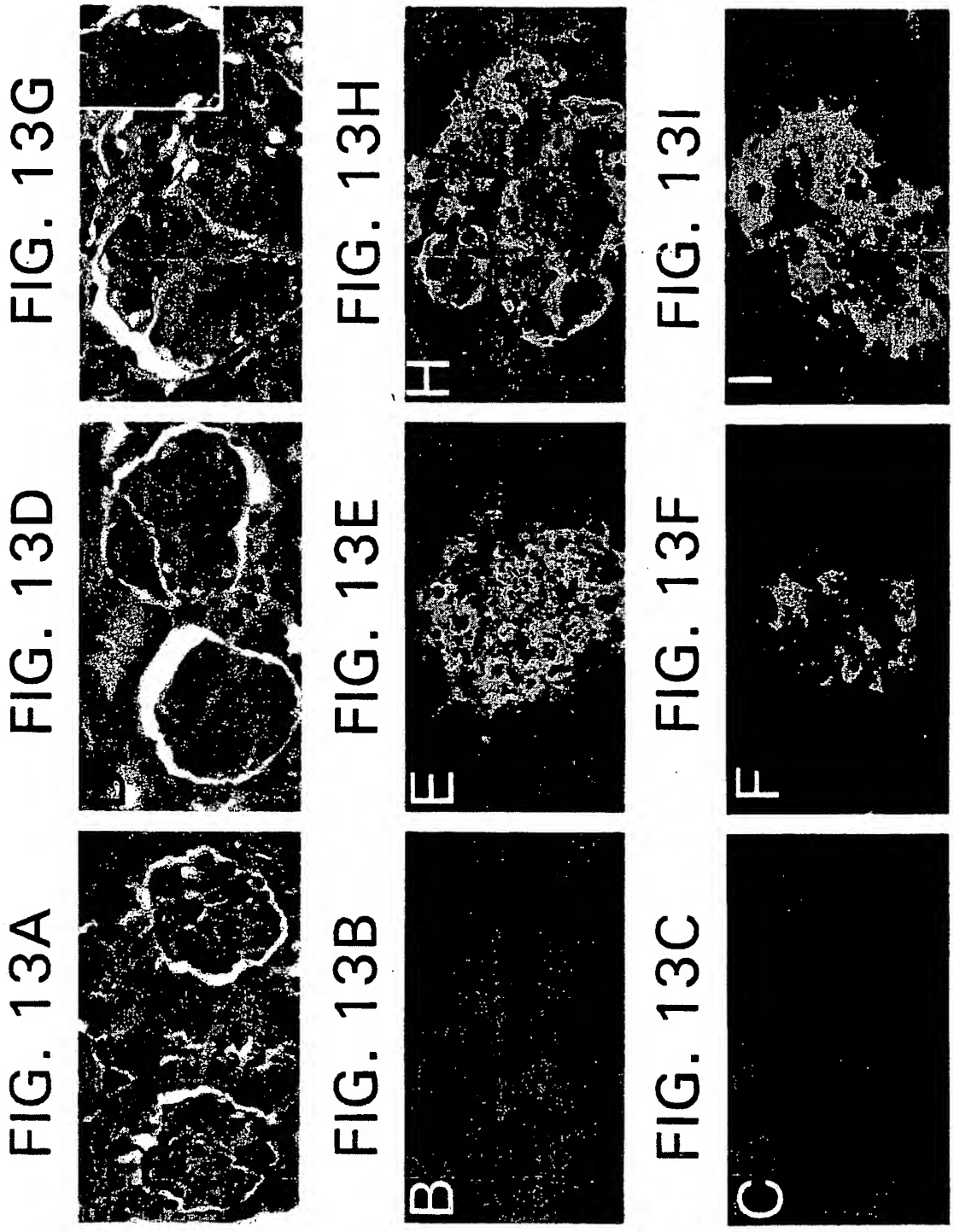


FIG. 13A
FIG. 13B
FIG. 13C
FIG. 13D
FIG. 13E
FIG. 13F
FIG. 13G
FIG. 13H
FIG. 13I
B
C
E
F
H
I

FIG. 14A

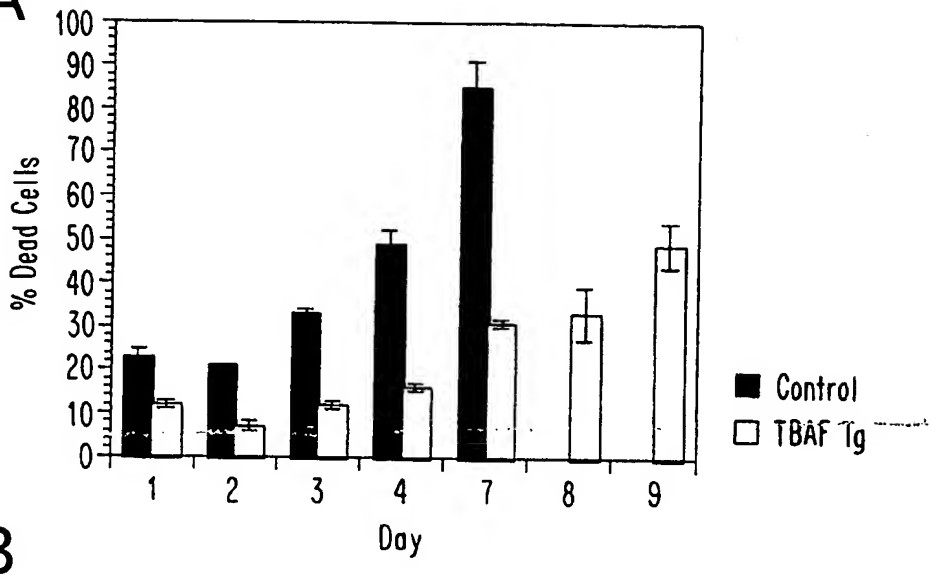


FIG. 14B

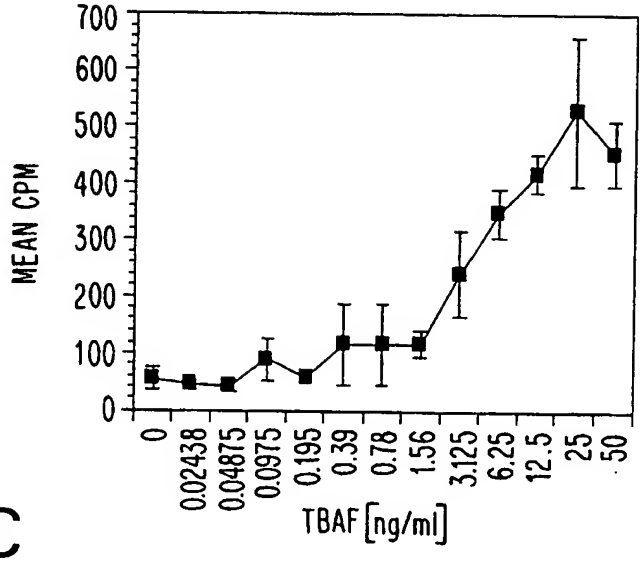
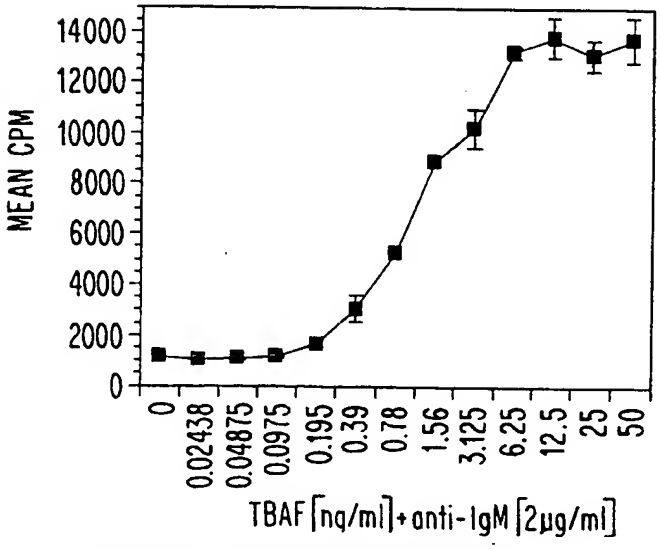


FIG. 14C



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/03653

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/705 C12N15/62 C07K19/00 C12N15/70
A61K48/00 A61K38/19 C07K16/22 C07K16/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 46686 A (AMGEN INC) 11 December 1997 (1997-12-11) page 3, line 25 -page 16, line 15	1,5-9, 18-21, 23-25, 28-33, 36,37
Y	WO 98 49305 A (AMGEN INC ;BOYLE WILLIAM J (US); WOODEN SCOTT (US)) 5 November 1998 (1998-11-05) page 4, line 8 -page 8, line 35; example 1	1,5-9, 18-21, 23-25, 28-33, 36,37
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 September 2000	02/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	EP 0 869 180 A (SMITHKLINE BEECHAM CORP) 7 October 1998 (1998-10-07) SEQ.ID.N.2 and 4 the whole document	1,2, 14-16
A	WO 98 55621 A (MASIAKOWSKI PIOTR ;REGENERON PHARMA (US); VALENZUELA DAVID (US)) 10 December 1998 (1998-12-10) SEQ.ID.N.4 and 6. the whole document	1,3, 14-16
A	EP 0 675 200 A (MOCHIDA PHARM CO LTD ;OSAKA BIOSCIENCE INST (JP)) 4 October 1995 (1995-10-04) SEQ.ID.N.3	3,14-16
A	YAMAGUCHI K ET AL: "Characterisation of structural domains of human osteoclastogenesis inhibitory factor" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 9, no. 273, 27 February 1998 (1998-02-27), pages 5117-5123, XP002077021 ISSN: 0021-9258 the whole document	1,14-16
A	DANILENKO D M (REPRINT) ET AL: "AGP - 1, a novel member of the tumor necrosis factor family, induces hepatic necrosis and inflammation in transgenic mice" FASEB JOURNAL,US,FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, BETHESDA, MD, vol. 11, no. 3, 28 February 1997 (1997-02-28), XP002045026 ISSN: 0892-6638 abstract	1
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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	SCHNEIDER P ET AL: "BAFF, A NOVEL LIGAND OF THE TUMOR NECROSIS FACTOR FAMILY, STIMULATES B CELL GROWTH" JOURNAL OF EXPERIMENTAL MEDICINE,TOKYO,JP, vol. 189, no. 11, 7 June 1999 (1999-06-07), pages 1747-1756, XP000915409 ISSN: 0022-1007 the whole document	14-17
E	WO 00 24782 A (AMGEN INC) 4 May 2000 (2000-05-04) the whole document	1,6-13

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 6-9

Present claims 6-9 relate to an extremely large number of possible compounds. In fact, the claims contain so many options, that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely immunoglobulin Fc-regions as those mentioned in the description at pages 18-20.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/03653

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